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14. ABSTRACT
One of the most persistent factors accounting for the continuing mortality in cancer patients is the development of multidrug resistance (MDR). In this study, we have shown that over expression of glucosylceramide synthase (GCS) is not a consistent characteristic in breast cancer cells. We have determined that in order for GCS to be over expressed, cells must be selected to grow in the presence of anticancer agents that activate ceramide formation. This abundance of ceramide enhances GCS message which results in increases in glucosylceramide (GC). We found this to be the case with Adriamycin but not with cisplatin or etoposide. This study also showed that limiting GCS activity down regulated the expression of MDR1. In assessing P-gp status in the MCF-7-AdrR cells transfected with GCS antisense, we observed a dramatic decrease in the level of MDR1 expression, (80% down by RT-PCR), which translated into a similar decrease in P-gp protein levels (Western-blot). These findings demonstrated an interesting yet ambiguous relationship between GCS, which regulates ceramide metabolism, and the expression of P-gp. To gain insight into the relationship of GCS and MDR1, we studied the influence of sphingolipids on MDR1 expression. When cells, MCF-7 and MDA-MB-231, challenged with high levels of ceramide utilize a glycosylation route to limit ceramide's residence time, this action promotes enhanced expression of the multidrug resistant phenotype in cancer cells through what we propose is a GC intermediate.

15. SUBJECT TERMS
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INTRODUCTION

Poor response to chemotherapy is a major clinical problem, and in most instances drug resistance is the underlying cause (1). This is a most undesirable situation, and patients and oncologists would welcome its possible correction. In breast cancer nearly 50% of patients demonstrate primary and/or secondary resistance to Adriamycin (doxorubicin) (2). Several mechanisms of drug resistance are being examined, and avenues to reverse resistance are being sought. Research strategies in this area have become increasingly aimed at molecular targets such as P-glycoprotein (P-gp), multidrug resistance associated protein (MRP), topoisomerase, and Bcl-2 protooncogene, to name a few. Overexpression of the membrane efflux transporter, P-gp, is one of the most consistent biological alterations in drug resistance (1,3). P-gp (170 kDa), the product of the *MDR1* gene, is an energy-dependent pump that reduces the intracellular concentration of specific anticancer drugs, and it has been studied extensively. Our approach to drug resistance involves ceramide metabolism. Ceramide, a neutral lipid, has been shown to play a role in signaling apoptosis (4). Ceramide can be generated by two different pathways. The first one is the *de novo* pathway, whereby serine and palmitoyl-CoA condense through a series of reactions; generate dihydroceramide and ceramide, respectively. The second one is the salvage pathway, which involves the hydrolysis of membrane sphingomyelin (SM) by sphingomyelinase (SMase). Once ceramide is generated, it can be converted into a number of other bioactive sphingolipids, including ceramide-1-phosphate (by ceramide phosphorylation via ceramide kinase), glucosylceramide (by ceramide glycosylation via glucosylceramide synthase), and sphingosine (by ceramide hydrolysis via ceramidase). Several front-line anticancer agents elicit the formation of ceramide (4,5). If ceramide formation in response to drug treatment is blocked, then the cytotoxic impact of the drug is largely reduced (6,7). This demonstrates ceramide’s role in drug responses. Ceramide added directly to cells circumvents the enzyme route of ceramide formation and promotes an apoptotic cascade directly (7,8). Our group showed that increased cellular capacity for ceramide glycosylation, catalyzed by glucosylceramide synthase (GCS), is associated with chemotherapy resistance in cancer cells (9-13). In cultured breast cancer cells, sensitivity to anthracyclines and taxanes can be decreased or increased simply by manipulation of GCS activity (11,14,15). For example, transfection of drug sensitive MCF-7 breast cancer cells with GCS cDNA confers resistance to Adriamycin (14), and transfection of multidrug resistance MCF-7-AdrR breast cancer cells with antisense GCS (asGCS) increases cell sensitivity to chemotherapy by a factor of 28-fold for Adriamycin, more than 100-fold for Vinblastine, and more than 200-fold for paclitaxel (11).

The objectives of this study were : 1) to determine whether overexpression of GCS was a characteristic of chemotherapy resistance in breast cancer cells; 2) to determine whether anticancer agents influenced the expression the expression of GCS; 3) to determine whether overexpression of GCS and overexpression of P-glycoprotein were allied or dissociated events in drug resistance in breast cancer.

BODY

Deviations from the original statement of work
The aim of the study is to understand the role of glucosylceramide synthase (GCS) in the mechanism of drug resistance in breast cancer. We used a slightly different approach than we planned when we wrote the project, namely, we incorporated the use of another cell line, MCF-7-AdrR/asGCS. This cell line is an Adriamycin-resistant breast cancer cell line stably transfected with GCS antisense. In this cell line, GCS activity is knocked down by 30%. This tool helped us to understand the implications of GCS in the mechanisms of drug resistance in breast cancer cells, in particular as regards P-gp. This research allowed us to make important discoveries on the connection between GCS and P-gp, and this work has been reported in a publication (16). Moreover, this work can be related to task 3 of the statement of work: “Determine whether overexpression of GCS and overexpression of P-gp are allied or dissociated”.

ACCOMPLISHMENTS

Task 1: Determine whether overexpression of GCS is characteristic of chemotherapy resistance in breast cancer.

a. Measure GC mass and GCS mRNA in wild-type and in drug resistant cells.
For this study we used several breast cancer cell lines:

1. Adriamycin-resistant cells, MCF-7-AdrR and the matched wild type MCF-7.
2. Cisplatin-resistant cells, MCF-7/CDDP, and the matched wild-type, MCF-7/P.
3. Etoposide-resistant cells, MCF-7/VP, and the matched wild-type, MCF-7/F.

The levels of GCS mRNA were surveyed by real-time RT-PCR. The highest GCS expression was observed in MCF-7-AdrR cells compared to wild-type breast cancer cells, whereas a GCS expression in MCF-7/CDDP cells was not significantly different compared to MCF-7/P. A decrease in GCS mRNA was observed in MCF-7/VP cells compared to MCF-7/F cells.

In Figure 1, GCS mRNA was 1.8- and 1.2-fold higher in MCF-7-AdrR and MCF-7/CDDP cells, respectively, compared to their wild-types.
GCS expression in etoposide resistant cells, MCF-7/VP, was lowered by 36% compared to MCF-7/F.

For GC quantitation, total lipids were extracted and analyzed by thin-layer chromatography (TLC) using a solvent system containing chloroform/ methanol/NH_4OH (70:20:4, v/v/v). Approximately 250 µg of lipid were loaded per lane. After development, TLC plates were sprayed with sulfuric acid and heated (30 min, 180 C). The GC chars were quantitated by scanning densitometry, using a GC standard curve (0.5-5.0 µg). Figure 2 showed that GC mass was 2-fold higher in MCF-7-AdrR cells compared to the wild-type counterpart (4.0 ± 1.0 versus 2.02 ± 0.58 µg GC). No significant differences were found in MCF-7/CDDP compared to wild-type counterpart (0.8 ± 0.1 versus 0.4 ± 0.3 µg). We found that GC mass decreased by 40 in etoposide-resistant cells compared to the wild-type cells.

b. Determination of ceramidase expression in MCF-7 and drug resistant MCF-cells. Another major avenue of ceramide elimination is by hydrolysis via ceramidase (CDase). Ceramidases hydrolyze ceramide into sphingosine, a precursor of the antiapoptotic factor sphingosine-1-phosphate, and free fatty acid. The acid ceramidase is located within lysosomes, neutral ceramidase is localized mainly in plasma membrane, and the alkaline ceramidase is in the Golgi apparatus.

In Figure 3, we assessed ceramidase expression in MCF-7 cells and in drug resistant MCF-7 variants (by real-time RT-PCR). MCF-7/VP showed a 2-fold increase in alkaline CDase expression compared to MCF-7 cells. A 3-fold increase was shown for MCF-7/CDDP cells compared to their wild-type MCF-7/P. In MCF-7/AdrR compared to MCF-7 cells, levels of expression of alkaline CDase did not change, whereas acid CDase expression decreased by 2.6-fold. The major difference between MCF-7/AdrR and MCF-7 was the neutral CDase, which was 3.4-fold higher in drug resistant cells.

Task 2: Determine whether anticancer agents influence the expression of GCS.

a. Determination of whether ceramide and glucosylceramide generation are influenced by paclitaxel, cisplatin, Adriamycin, etoposide, or SDZ PSC 833 in breast cancer cells. MCF-7 cells were treated separately with Adriamycin (2.5 μM), cisplatin (CDDP) (10 μM), etoposide (VP16) (1.0 μM), paclitaxel (1.5 μM), and SDZ PCS 833 (5.0 μM) for 30 min, 4 and 24 h in medium containing [^{3}H] palmitate as metabolic tracer. In MCF-7 cells, CDDP and VP16 did not induce ceramide generation at any treatment time. We observed that paclitaxel induced a significant 2-fold increase in ceramide generation after 24 h. Adriamycin generated an 83% elevation in ceramide at 24 h. With SDZ PSC 833 ceramide production increased by 2-, 3.9-, and 9-fold at 30 min, 4, and 24 h, respectively (Figure 4A), illustrating a time-dependent response. Only drugs that elicit a significant increase in ceramide induced GC production. GC production increased 54% after 24 h treatment with paclitaxel, and SDZ PCS 833 induced a 2-fold increase at 4, and 24 h (Figure 4B). Even though Adriamycin generated ceramide at 24 h in MCF-7 cells, GC production was not observed.

To determine whether the induction of ceramide by chemotherapy drugs in breast cancer cells was cell-type dependent, we assessed ceramide and GC production in another breast cancer cell line, MDA-MB-231 (ER(-) cell line).

MDA-MB-231 cells demonstrated similar responses compared to MCF-7 cells regarding ceramide and GC production via paclitaxel, cisplatin, Adriamycin, etoposide, and SDZ PSC 833 exposure (Figure 5). At 30 min, only SDZ PSC 833 increased ceramide generation (+72%) compared to untreated cells. SDZ PSC 833 continued to promote time-dependent in ceramide 2.6- and 4.6-fold at 4 and 24 h, respectively (Figure 5A). MDA-MB-231 cells responded to SDZ PSC 833 with significant increases in GC levels at 4 (+267%) and 24 h (+307%) (Figure 5B). Increases in ceramide (+192%) and GC (+171%) were also found in MDA-MB-231 cells treated with paclitaxel (24 h), and Adriamycin induced a 2.3-fold increase in ceramide production at 24 h without any elevation in GC level.

b. Determine whether anticancer drugs enhance GCS promoter activity in MCF-7 cells. First, we determined the IC_{50} (concentration of drug affecting a 50% kill) of epirubicin and idarubicin in MCF-7 cells. This was essential for subsequent work with the GCS promoter and possible activation by epirubicin and idarubicin.

IC_{50} for idarubicin and epirubicin in MCF-7 cells was 0.17 and 0.3 µM, respectively (data not shown).

MCF-7 cells were transiently transfected with the luciferase-reporter plasmid of the full-length sequence of the human GCS promoter. Cells were then treated with either C6- ceramide (5.0 µM), epirubicin (0.5 µM), or Adriamycin (0.5 µM) for 4 and 48 hr. After 4 hr, luciferase activity in cells treated with C6-ceramide showed a 1.2-fold increase compared to ceramide-naïve cells. Idarubicin, epirubicin, and Adriamycin did not activate the GCS promoter at 4 hr (Figure 6A).

After 48 hours, C6-ceramide, epirubicin, and Adriamycin induced a GCS promoter activation of 4-, 2.4-, and 1.8-fold, respectively (Figure 6B).

c. Evaluate levels of GCS mRNA in MCF-7 cells after exposure to chemotherapy drugs. Initial cell viability experiments were carried out to determine the IC_{50} doses for Adriamycin and taxol. Values obtained with Adriamycin and taxol were 0.41 and 0.09 µM, respectively, in MCF-7 cells (data not shown).

MCF-7 cells were treated with drugs such as etoposide, N-(4-hydroxyphenyl) retinamide (4-HPR), Adriamycin, and CDDP. After 48 hr exposure, only Adriamycin induced an increase (2.4-fold) in GCS expression compared to untreated MCF-7 cells (Figure 7). Adriamycin was the only ceramide generating drug in our model (see figure 4).

d. Determine whether C6-ceramide can act directly to enhance GCS transcription. MCF-7 cells were incubated with C6-ceramide (5.0 µM) for 48 hr. This short-chain analog of long-chain, natural ceramide is cell permeable and potentiates biological responses seen with ceramide-generating chemotherapeutic agents.

Figure 8 shows that C6-ceramide induced a 9-fold activation of GCS expression. The C6-dihydro-ceramide (C6-di-cer) used as negative control, has no effect on GCS message expression.

e. Cellular metabolism of short-chain ceramide. To obtain a more detailed analysis of ceramide metabolism in breast cancer cells, including possible conversion to sphingomyelin (SPM) and lactosylceramide (LacCer), we exposed MCF-7 and MDA-MB-231 cells to [^{14}C]C6-cer. The [^{14}C]C6-cer was diluted with unlabeled C6-cer to obtain a concentration in the medium of 5.0 µg/ml. In Figure 9, MDA-MB-231 cells converted 80% of the counts taken up to [^{14}C]C6-GC after 24 h, and MCF-7 cells show a conversion rate of 62%. Both cell lines synthesized [^{14}C]C6-LacCer up to 10% of the total counts. MCF-7 and MDA-MB-231 cells synthesized C6-SPM from [^{14}C]C6-cer, with counts ranging from 8 to 18% of the total radioactivity.

f. Metabolism of [^{14}C]C6-ceramide by drug resistant breast cancer cells. We exposed the various drug resistant cell lines to [^{14}C]C6-cer at a low concentration (0.2 µg /ml). MCF-7, MCF-7/VP, MCF-7/CDDP, and MCF-7/AdrR

cells took up 14.3, 14.2, 19, and 10.2% of [^{14}C]C6-cer, respectively, and converted 49, 76, 95, and 37% of that to short-chain sphingomyelin (Figure 9). Therefore, when cells are not challenged with excessive amounts of ceramide (as in the 5µg/ml regimen), the conversion to short-chain GC is very low.

Task 3: Determine whether overexpression of GCS and overexpression of p-glycoprotein are allied or dissociated.

Previously, we showed that GCS antisense transfection of multidrug-resistant MCF7-AdrR cells enhanced cell sensitivity to Adriamycin, Vinblastine, and paclitaxel (11).

a. **Influence of asGCS transfection on paclitaxel-induced ceramide accumulation.** Using mass analysis, we found that ceramide production in response to paclitaxel (1.0 µmol/L) was enhanced 3-fold in MCF-7-AdrR/asGCS cells compared with parental MCF-7-AdrR cells after 24 hour treatment (Figure 11). Ceramide mass in both MCF-7-AdrR control and MCF-7-AdrR paclitaxel treated was 1.6 µg. Ceramide mass in MCF-7-AdrR/asGCS control and MCF-7-AdrR/asGCS paclitaxel treated was 1.0 and 3.25 µg, respectively.

   This shows that ceramide glycosylation is retarded by antisense GCS (because of high free ceramide levels). To further assess the influence of GCS antisense transfection on cell response to chemotherapy, we next measured uptake and efflux variables of paclitaxel.

b. **Chemotherapy uptake in MCF-7-AdrR and MCF-7-AdrR/asGCS cells.** In Figure 12, experiments with radiolabeled chemotherapy drugs showed that after 60 minutes, intracellular levels of paclitaxel were 8.6-fold greater in MCF-7-AdrR/asGCS cells compared with MCF-7-AdrR cells. This means that either drug uptake is enhanced or efflux is subdued.

c. **P-gp expression in MCF-7-AdrR and MCF-7-AdrR/asGCS cells.** Because of pronounced differences in drug levels in the two cell lines, we assessed P-gp expression by mRNA and protein determinations. Figure 13 shows that the level of MDR1 mRNA, evaluated by reverse RT-PCR was dramatically lower in MCF-7-AdrR/asGCS cells compared with MCF-7-AdrR cells. We confirmed this by Western blot. Whereas MCF-7-AdrR cells contained characteristically elevated levels of P-gp, MCF-7-AdrR/asGCS cells displayed a dramatic decrease (~80%) in P-gp levels.

d. **Influence of PPMP and GCS siRNA on MDR1 gene expression in MCF-7-AdrR cells.** To determine whether decreased MDR1 expression in asGCS cells was really due to GCS inhibition and/or downregulation, we evaluated the influence of D-L-*threo*-PPMP, a chemical inhibitor of GCS, on MDR1 expression in MDR1-rich MCF-7-AdrR cells. PPMP greatly diminished the expression of MDR1 in MCF-7-AdrR cells, with demonstrated stereospecificity. Unlike D-L-*threo*-PPMP, D-L-*erythro*-PPMP is not a GCS inhibitor, and this stereoisomer had no influence on MDR1 expression (Figure 14A).

   Real-time RT-PCR showed that MDR1 expression in MCF-7-AdrR cells treated with D-L-*threo*-PPMP and D-L-*erythro*-PPMP was reduced by 58% and 12%, respectively, compared with untreated MCF-7-AdrR cells (Figure 14B).

To reinforce the results obtained with PPMP and to confirm that changes in MCF-7-AdrR/asGCS cellular MDR1 expression were not due to clonal artifacts, we used GCS siRNA to treat MCF-7-AdrR cells. siRNA was introduced into cells using lipofectAMINE in serum-free medium for 4 hours. After 48 hours, both GCS and MDR1 mRNA were dramatically decreased by GCS siRNA compared with lipofectAMINE only controls (Figure 14C). The siRNA had no effect on expression levels of -actin.

e. Influence of C8-ceramide (C8-cer) and glucosyl C8-ceramide (C8-GC) on MDR1 expression in MCF-7 and MDA-MB-231 cells. To determine whether ceramide and GC could influence MDR1 expression, we treated MCF-7 and MDA-MB-231 cells with short-chain analogs C8-cer and C8-GC. Acute exposure to C8-cer did not alter MDR1 expression in MCF-7 cells, whereas exposure of MDA-MB-231 cells to C8-cer (5.0 µg/ml) was cytotoxic (data not shown). We then investigated whether GC, a known P-gp substrate, would regulate MDR1 expression. Acute exposure of MCF-7 and MDA-MB-231 cells to C8-GC (72 h) resulted in 2- and 4-fold increases, respectively, in MDR1 mRNA levels (Figure 15).

f. Ceramide influence on MDR1 phenotype. The data of Figure 16 demonstrate the high capacity of MDA-MB-231 cells to generate [^{14}C]GC from [^{14}C]C6-cer supplements. We used this model to assess whether ceramide via conversion to GC would influence expression of MDR1. MDA-MB-231 cells were first grown with 2.5 µg/ml doses of C8-cer for two passages, after which the dose was increased to 5.0 µg/ml, with minimal cytotoxicity. Prolonged growth of MDA-MB-231 cells in C8-ceramide-containing medium caused a robust increase in MDR1 mRNA levels. Figure 16A demonstrates, by gel electrophoresis of RT-PCR products, the dramatic increase in the level of MDR1 expression in MDA-MB-231/C8cer cells (at passage 22) compared to wild-type MDA-MB-231 cells. In order to determine if upregulation of MDR1 mRNA levels resulted in enhanced expression of P-gp protein, Western blot analyses were conducted. The results of figure 16B show that under the conditions employed, P-gp was undetectable in wild-type MDA-MB-231 cells; however, P-gp levels increased with increased C8-ceramide exposure time (passages 12 and 22). These data show that C8-ceramide exposure upregulates both MDR1 mRNA and protein.

g. Resistance to Adriamycin and paclitaxel in MDA-MB-231/c8cer cells compared to MDA-MB-231 cells. Chemotherapy sensitivity status of MDA-MB-231/C8cer cells was evaluated and compared with wild-type MDA-MB-231 cells. Adriamycin and paclitaxel, natural product chemotherapy drugs that are substrates for P-gp, were used. MDA-MB-231/C8cer cells were more resistant to anticancer drugs. As shown by dose-response/cell viability curves (Figure 17), MDA-MB-231/C8cer cells were approximately 3- and 9-fold more resistant to Adriamycin and paclitaxel, respectively, compared to MDA-MB-231 cells. Values of IC_{50} were 88 and 270 nM for Adriamycin in MDA-MB-231 and MDA-MB-231/C8cer cells, respectively, and 36 and 331 nM for paclitaxel in MDA-MB-231 and MDA-MB-231/C8cer cells, respectively.

KEY RESEARCH ACCOMPLISHMENTS

Determine GC mass and GCS mRNA in wild-type and in drug resistant cells.

Determine ceramidase expression in MCF-7 and in drug resistant MCF-7 cells.

Determine that ceramide and glucosylceramide generation in breast cancer cells are influenced by paclitaxel, Adriamycin, or SDZ PSC833 but not by cisplatin, or etoposide.

Determine that anticancer drugs (Adriamycin, idarubicin, and epirubicin) and c6-ceramide enhanced GCS promoter activity in MCF-7 cells.

Determine that Adriamycin in MCF-7 cells induced GCS mRNA but not etoposide, 4-HPR, or cisplatin.

Determine that c6-ceramide directly enhanced GCS transcription.

Determine cellular metabolism of short-chain ceramide in MCF-7 and MDA-MB-231 cells.

Determine cellular metabolism of short-chain ceramide by drug resistant breast cancer cells.

Determine that MCF-7-AdrR cells transfected with GCS antisense showed enhanced ceramide formation compared with MCF-7-AdrR cells when challenged with paclitaxel.

Determine that paclitaxel uptake increased in MCF-7-AdrR/asGCS compared to MCF-7-AdrR cells.

Determine that P-glycoprotein expression decreased in MCF-7-AdrR/asGCS cells compared to MCF-7-AdrR cells.

Determine that PPMP and GCS siRNA decreased MDR1 gene expression in MCF-7-AdrR cells.

Determine that c8-glucosylceramide increased MDR1 mRNA levels in MCF-7 and MDA-MB-231 cells.

Determine that ceramide influenced MDR1 phenotype.

Determine that MDA-MB-231/c8cer cells (prolonged growth of MDA-MB-231 cells in c8-ceramide-containing medium) were more resistant to Adriamycin and paclitaxel compared to MDA-MB-231 cells.

REPORTABLE OUTCOMES

Posters:

1. “Relationship between Glucosylceramide synthase and P-glycoprotein in drug resistant human breast cancer cells.” Era of hope 2005, Department of Defense Breast Cancer Research Program. Philadelphia, Pennsylvania, June 8-11, 2005.
2. “Sphingolipids can influence breast cancer cell phenotype”. 4th International Charleston Ceramide Conference. Pacific Grove, California, March 7-11, 2007.

Oral Presentation

“Glucosylceramide synthase blockade downregulates P-glycoprotein and resensitizes multidrug-resistant breast cancer cells to anticancer drugs.” Third International Charleston Ceramide Conference. Charleston, SC, March 2-6, 2005.

Publications

1. "Glucosylceramide synthase blockade downregulates P-glycoprotein and resensitizes multidrug-resistant breast cancer cells to anticancer drugs." Gouazé V, Liu YY, Prickett CS, Yu JY, Giuliano AE, Cabot MC. Cancer Res., 2005, 65:3861-7.
2. "Ceramide and glucosylceramide upregulate expression of the multidrug resistance gene MDR1 in cancer cells." Gouazé-Andersson V, Yu JY, Kreitenberg AJ, Bielawska A, Giuliano AE, Cabot MC. Biochim Biophys Acta, 2007, 1771:1407-17.

KEY PERSONNEL

1. Valérie Gouazé-Andersson, PhD – Principal Investigator
2. Myles C. Cabot, PhD – Mentor

CONCLUSION

This study demonstrated that overexpression of GCS is not a consistent characteristic of chemotherapy resistance in breast cancer. We have determined that in order for GCS to be overexpressed, cells must be selected to grow in the presence of anticancer agents that activate ceramide formation. This abundance of ceramide enhances GCS message which results in increases in glucosylceramide. We found this to be the case with Adriamycin but not with cisplatin or etoposide. This study also showed that limiting GCS activity down-regulated the expression of MDR1, and that high levels of ceramide can enhance expression of the multidrug resistant phenotype in cancer cells through what we propose is glucosylceramide intermediate.

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APPENDICES

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Ceramide and glucosylceramide upregulate expression of the multidrug resistance gene MDR1 in cancer cells

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Abstract

In the present study we used human breast cancer cell lines to assess the influence of ceramide and glucosylceramide (GC) on expression of *MDR1*, the multidrug resistance gene that codes for P-glycoprotein (P-gp), because GC has been shown to be a substrate for P-gp. Acute exposure (72 h) to C8-ceramide (5 μg/ml culture medium), a cell-permeable ceramide, increased MDR1 mRNA levels by 3- and 5-fold in T47D and in MDA-MB-435 cells, respectively. Acute exposure of MCF-7 and MDA-MB-231 cells to C8-GC (10 μg/ml culture medium), a cell-permeable analog of GC, increased MDR1 expression by 2- and 4- fold, respectively. Chronic exposure of MDA-MB-231 cells to C8-ceramide for extended periods enhanced MDR1 mRNA levels 45- and 390-fold at passages 12 and 22, respectively, and also elicited expression of P-gp. High-passage C8-ceramide-grown MDA-MB-231 (MDA-MB-231/C8cer) cells were more resistant to doxorubicin and paclitaxel. Incubation with [1-^{14}C]C6-ceramide showed that cells converted short-chain ceramide into GC, lactosylceramide, and sphingomyelin. When challenged with 5 μg/ml [1-^{14}C]C6-ceramide, MDA-MB-231, MDA-MB-435, MCF-7, and T47D cells took up 31, 17, 21, and 13%, respectively, and converted 82, 58, 62, and 58% of that to short-chain GC. Exposing cells to the GCS inhibitor, ethylenedioxy-P4, a substituted analog of 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol, prevented ceramide's enhancement of MDR1 expression. These experiments show that high levels of ceramide and GC enhance expression of the multidrug resistance phenotype in cancer cells. Therefore, ceramide's role as a messenger of cytotoxic response might be linked to the multidrug resistance pathway.

Keywords: Multidrug resistance; Ceramide; Glucosylceramide; P-glycoprotein; Breast cancer

1. Introduction

Cancer cells develop various mechanisms to escape the cytotoxic impact of chemotherapy drugs. These cellular changes present a formidable barrier to the successful treatment of cancer. One of the most consistent biochemical alterations in drug resistance is the overexpression of P-glycoprotein (P-gp). P-gp is a membrane-resident protein that functions as an energy-dependent pump reducing the intracellular concentration of anticancer drugs, chiefly hydrophobic, natural product agents such as anthracyclines, *Vinca* alkaloids, and the taxane, paclitaxel [1]. The *MDR1* gene, responsible for multidrug resistance, codes for P-gp [2].

P-gp and related transporter proteins reduce the intracellular concentration of antitumor, antiviral, and antibacterial agents and thus impede treatment of cancer, HIV/AIDS, and bacterial infections. ABC transporter proteins also play a role in *Plasmodium falciparum* resistance to antimalarials [3], and resistance of *Leishmania* to antimonials [4]. It is thus imperative not only to develop and test novel ABC-transporter antagonists such as

Abbreviations: ABC, ATP-binding cassette protein; CTP, cytidine triphosphate; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; GC, glucosylceramide; GCS, glucosylceramide synthase; IC_{50}, concentration of agent that inhibits 50% of cell growth; *MDR1* gene, multidrug resistance gene 1; P-gp, P-glycoprotein; ethylenedioxy-P4, ethylenedioxy-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol; qPCR, quantitative real-time RT-PCR; RT-PCR, reverse transcriptase polymerase chain reaction; TLC, thin-layer chromatography; UDP, uridine diphosphate

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Biricodar (VX710) and LY335979, but also explore and identify regulatory junctures of these proteins.

Several reports have surfaced documenting the interesting but curious association of multidrug resistance with glycolipids [5–9]. Other works have demonstrated that glucosylceramide synthase (GCS) regulates cytotoxic response to chemotherapy [10–13]. GCS catalyzes glucosylceramide (GC) formation, using ceramide and UDP-glucose as reactants. In wild-type cancer cells, it was shown that pressure for resistance to natural product chemotherapy selected for enhanced GCS expression and activity, in addition to enhanced P-gp expression [5]; a complementary study in multidrug resistant cells showed that inhibition of GCS suppressed the expression of MDR1 [12]. These studies indicate that GCS serves a regulatory role in expression of the multidrug resistance phenotype.

The present study was undertaken to determine whether lipids of the ceramide metabolic pathway affect a cell's proclivity towards a multidrug-resistant phenotype. Here we show that ceramide (8-carbon and 6-carbon cell-permeable analogs) and a glycosylated counterpart, glucosyl-C8:0 ceramide (C8-GC), upregulate MDR1 expression in human breast cancer cell lines when supplemented to the growth medium; aliphatic lipids such as octanoic acid and oleic acid were not active. All cell lines examined readily glycosylated short-chain ceramide forming, in abundance, the corresponding short-chain GC, and the introduction of a GCS inhibitor, which abolished GC synthesis, prevented ceramide-enhanced MDR1 mRNA increases. Of particular significance to our study is work showing that hexanoyl-glucosylceramide (C6-GC) inhibits P-gp activity, and it is known that inhibitor/substrates of MDR1 can upregulate MDR1 expression [14]. Further, because GC has been shown to be a substrate for the flipase action of P-gp [15], our work suggests that build-up of cellular GC may lead to the induction of MDR1/P-gp. While the role of ceramide as a cellular messenger of apoptosis has been extensively studied [16,17], the possibility of simultaneous induction of MDR1 via GC is novel.

2. Materials and methods

2.1. Cell culture

MCF-7, T47D, MDA-MB-231 and MDA-MB-435 human breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in Gibco™ RPMI-1640 medium (Invitrogen Corp., Grand Island, NY) containing 10% FBS (HyClone, Logan, UT), 50 units/ml penicillin, 50 μg/ml streptomycin, and 584 mg/L-glutamine (in addition to the glutamine already in the medium formulation); cells were subcultured using Gibco's 0.05% trypsin/0.53 mM EDTA solution. Cells were grown in a humidified atmosphere, 95% air, 5% CO_2, at 37 °C and subcultured at confluence. K-B-Ch^R-8-5 (colchicine-resistant human epidermoid carcinoma) cells, used as a P-gp-positive control, were a gift from Dr. Michael M. Gottesman, Laboratory of Cell Biology, National Cancer Institute (Bethesda, MD). Cells were cultured in high glucose DMEM plus 10% FBS, with glutamine, penicillin and streptomycin as above, and colchicine (10 ng/ml).

[1-^{14}C]C6-ceramide (N [1-^{14}C]hexanoyl-sphingosine) was synthesized as described previously [18], and upon purification had a radiospecific activity of 135,118 cpm/nmol. The GCS inhibitor ethylenedioxy-P4 [19] is a phenyl ring substituted analog of parent P4, D-*threo*-1-phenyl-2-hexadacanoylamino-3-pyrrodilino-1-propanol, and was a gift from Dr. James Shayman, University of Michigan, Ann Arbor.

2.2. Cell supplements

C8-ceramide (made from D-*erythro*-sphingosine), C6-ceramide, D-*erythro*-sphingosine, and glucosyl-C8-ceramide (D-glucosyl-β1-1′-*N*-octanoyl-D-*erythro*-sphingosine) (Avanti Polar Lipids, Alabaster, AL) were dissolved in ethanol and added in microliter amounts to 37 °C complete culture medium to achieve the desired final concentration of lipid. [1-^{14}C]C6-ceramide was diluted with unlabeled C6-ceramide to achieve a radiospecific activity of 6756 cpm/nmol (16,890 cpm/μg), for cell metabolism experiments. Ethanol stock solutions of these lipids were stored at −20 °C. All lipid-containing media were prepared freshly and not reused. Exposure regimens are given in the figure legends.

2.3. Continuous growth of MDA-MB-231 cells with C8-ceramide

MDA-MB-231 cells (1.5×10^6) were seeded in T-75 flasks and grown to confluence (15×10^6 cells) over two passages in medium containing 2.5 μg/ml C8-ceramide before being switched to medium containing 5.0 μg/ml C8-ceramide. Cells were then continually passaged at 1:10 in medium containing 5.0 μg/ml C8-ceramide and used in experiments at various passages. Cells grown continuously in medium containing C8-ceramide were termed MDA-MB-231/C8cer cells. In one set of experiments, MDA-MB-231/C8cer cells, at passage 17, were grown without C8-ceramide for 10 passages and used in subsequent assays.

2.4. Lipid analysis

Total lipids were extracted from cells as previously described [11]. C8-GC and C6-GC (these lipids co-migrate), and natural cellular GC were resolved from other lipids by thin-layer chromatography (TLC) on Silica Gel G plates (Analtech, Newark, DE) in a solvent system containing chloroform/methanol/ammonium hydroxide (70:35:4, v/v). In this solvent system certain GC species containing hydroxylated aliphatic moieties and galactosylceramide will also migrate with or near GC. C8-lactosylceramide (LacCer) (Avanti Polar Lipids) was resolved in a solvent system containing chloroform/methanol/water (60:40:8, v/v), and C6-sphingomyelin (C6-SPM) (Matreya, Pleasant Gap, PA) was resolved in a solvent system containing chloroform/methanol/ammonium hydroxide (60/35/8, v/v). Short-chain and natural ceramides were resolved in a solvent system containing chloroform/acetic acid (90:10, v/v). Visualization of lipids was by sulfuric acid charring [12], or by iodine staining, when quantitative analysis was by liquid scintillation counting (LSC). In some instance with radiolabeled lipids, TLC analysis was done by zonal profile scan [20,21] by scraping 2–3 mm zones of silica from the plate, followed by LSC for analysis of radioactivity.

2.5. RNA isolation and RT-PCR

Total RNA was isolated from cultured cells as previously described [12], and analysis of gene expression by real-time RT-PCR (qPCR) was conducted as previously described [12]. We used a Rotor-Gene RG-3000 analyzer (Corbett Research, Westborough, MA). Primers and probe sequences for MDR1 were as follows: MDR1 forward 5′-GGTTTATAGTAGGATTTACACGTGGTTG-3′, MDR1 reverse 5′-AAGATAGTATCTTTGCCCAGACAGC-3′, and MDR1 probe 5′ FAM CTAACCCTTGTGATTTTGGCCATCAGTCC Tamra 3′. Human β-actin was used as endogenous control. Both systems employed SuperScript III Platinum One-Step qRT-PCR kits (Invitrogen, Carlsbad, CA). To quantitate the abundance of each *MDR1* mRNA, all qPCR runs were conducted with a standard curve comprising a dilution series of qPCR plasmid standards (Invitrogen, Carlsbad, CA) containing open reading frame from start to stop codon for the β-actin gene, ranging from 2×10^2 to 2×10^6 copies/ml. Data were collected from threshold cycle values using the automatic function of the Rotor-Gene software program. The total amount of mRNA of each gene in each sample was calculated as the mean of triplicate samples. Levels of *MDR1* mRNA were expressed as the ratio of the target gene to the control gene. Cellular RNA was also analyzed by thermocycling (Bio-Rad, MyCycler) as described previously [12], and the RT-PCR products were analyzed by 1% agarose gel electrophoresis stained with ethidium bromide.

2.6. Rhodamine and [^{3}H]paclitaxel uptake/ efflux assay

The rhodamine assay, a functional test for P-gp efflux activity, was performed as described previously [11]. Briefly, MDA-MB-231 cells and high passage MDA-MB-231/C8cer cells were harvested using trypsin and washed with RPMI-1640 medium. To halt P-gp activity during uptake, cells (2×10^6) were pretreated with cyclosporine A (10 μM) for 2 h. Cells were then exposed to 5 μM rhodamine 123 in 1.0 ml 5% FBS RPMI-1640 medium, with cyclosporine A present, for 60 min at 37 °C. After centrifugation at 500×*g* for 10 min, supernatants were discarded, and the cells were further washed twice in RPMI-1640 medium. Cellular uptake of rhodamine-123 was measured by adding 200 μl culture medium containing 0.02% SDS to the washed cells, and fluorescence was measured at $\lambda_{excitation}$ 485 nm/ $\lambda_{emission}$ 530 nm using the FL600 Fluorescent Microplate Reader (BioTek, Winooski, VT). For efflux measurements, 1.0 ml of 5% FBS RPMI-1640 medium without or with cyclosporine A was added, and the cells were incubated at 37 °C for another 60 min. After three washes, cell fluorescence was measured using SDS as above. The efflux was calculated by the difference in cell fluorescence after the 60-min incubation compared with initial cell uptake.

Cellular uptake and efflux of paclitaxel was conducted as previously described [12] using [^{3}H]paclitaxel, 25.2 Ci/mmol (Moravek Biochemical, Brea, CA). Unlabeled and radiolabeled paclitaxel were mixed to achieve the desired radiospecific activity of 600 cpm/pmol.

2.7. Chemotherapy cytotoxicity assay — cell viability

Assays were performed as described previously [11]. MDA-MB-231 and MDA-MB-231/C8cer cells were seeded in 96-well plates (perimeter wells contain water) at 3,000 cells/0.1 ml/well, in 10% FBS RPMI-1640 medium and grown at 37 °C for 24 h before adding drug. Doxorubicin and paclitaxel, obtained from LKT Laboratories (St. Paul, MN), were added in 0.1 ml FBS-free medium, and the cells were cultured at 37 °C for 72 h. Cytotoxicity was determined using the CellTiter 96 Aqueous One Solution cell proliferation assay kit (Promega, Madison, WI). Absorbance at 490 nm was recorded using a Microplate Fluorescent Reader FL600.

2.8. Sphingomyelinase treatment

Cellular conversion of [^{14}C]C6-ceramide to SPM was confirmed by sphingomyelinase treatment. Following exposure (24 h) of cells to [^{14}C]C6-ceramide (84,500 cpm/ml medium) plus 4.75 μg/ml unlabeled C6-ceramide, total cellular lipids were extracted and chromatographed (TLC). The area of the plate corresponding to SPM (natural SPM from egg and commercial C6-SPM have similar R_f values) was scraped, and the radiolabeled lipid was removed from the Silica Gel by extraction with organic solvents [11]. After evaporation of solvent, the resultant lipid was suspended by sonication in 0.5 ml Tris–HCl buffer (pH 7.4, 0.1 M) containing 10 mM $MgCl_2$, and 0.2 U *B. cereus* sphingomyelinase was added and incubated with shaking in a 37 °C water bath for 30 min. The reaction mixture was extracted [11] and the presence of [^{14}C]C6-ceramide, the sphingomyelinase product, was verified by TLC.

2.9. Western blot for P-gp

Western blots were performed as previously described [12], using C219 murine monoclonal antibody (2.5 μg/ml) (Calbiochem, Pasadena, CA) against human P-gp.

3. Results

3.1. Ceramide and GC influence on MDR1 expression — acute exposure

Exposure of estrogen receptor-positive T47D cells, in mid-log phase growth, to C8-ceramide (5 μg/ml, 11.7 μM) for 72 h resulted in a 3-fold increase in MDR1 mRNA levels, over the control, ceramide-naïve cells (Fig. 1A). C8-ceramide also elicited

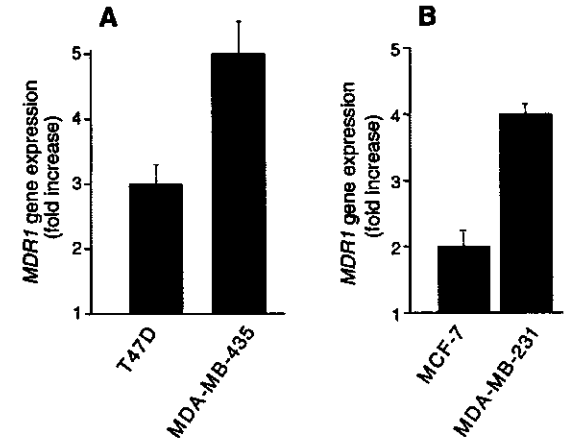

Fig. 1. Influence of C8-ceramide and C8-GC on MDR1 expression in human breast cancer cells. Cells (500,000) were seeded into 6-cm dishes and the following day supplemented with (A) C8-ceramide (5 μg/ml medium) or (B) C8-GC (10 μg/ml medium). Controls received ethanol vehicle (0.1% final concentration). After 72 h, total RNA was extracted and analyzed by qPCR. Fold increases in MDR1 were calculated using actual number of gene copies per unit β-actin expression. Results are the average of triplicate experiments, and experiments were repeated several times.

an increase in MDR1 mRNA levels in estrogen receptor-negative MDA-MB-435 cells; a 72 h exposure resulted in a near 5-fold enhancement (Fig. 1A). Acute exposure to C8-ceramide did not alter MDR1 expression in MCF-7 cells, whereas exposure of MDA-MB-231 cells to C8-ceramide (5 μg/ml) was cytotoxic and lowered in subsequent experiments. We then investigated whether GC, a known P-gp substrate [15], would upregulate MDR1 expression. Acute exposure of MCF-7 and MDA-MB-231 cells to C8-GC (72 h) resulted in 2- and 4-fold increases, respectively, in MDR1 mRNA levels (Fig. 1B). T47D and MDA-MB-435 cells did not respond to C8-GC (5 and 10 μg/ml) (data not shown).

Uptake and conversion of C8-ceramide to C8-GC was evidenced by TLC analysis of T47D and MDA-MB-435 cells that had been exposed to C8-ceramide (Fig. 2A). As shown by the chromatogram, both cell lines supplemented with C8-ceramide contained C8-GC, which migrated slightly below the natural, endogenous GC doublet. C8-GC was absent in control (−C8) T47D and MDA-MB-435 cells. Of note, both T47D and MDA-MB-435 cells responded to C8-ceramide with increased MDR1 mRNA levels (Fig. 1A); however, these cell lines did not respond to C8-GC when given as a supplement. Experiments to evaluate uptake of C8-GC revealed that all cell lines took up the short-chain cerebroside analog, and this is shown using two cell lines, MCF-7 and MDA-MB-231 as examples (Fig. 2B, C).

In order to determine whether responses were lipid-specific, cells were exposed to oleic acid and to octanoic acid, the fatty acid hydrolysis product of C8-ceramide. MDA-MB-435 cells, which were responsive to C8-ceramide (Fig. 1), were used in these experiments. Exposure of cells to 5 μg/ml of either fatty acid (72 h) failed to significantly alter MDR1 mRNA levels (qPCR), compared to unsupplemented cells. For example, using the actual number of gene copies per unit β-actin, octanoic acid

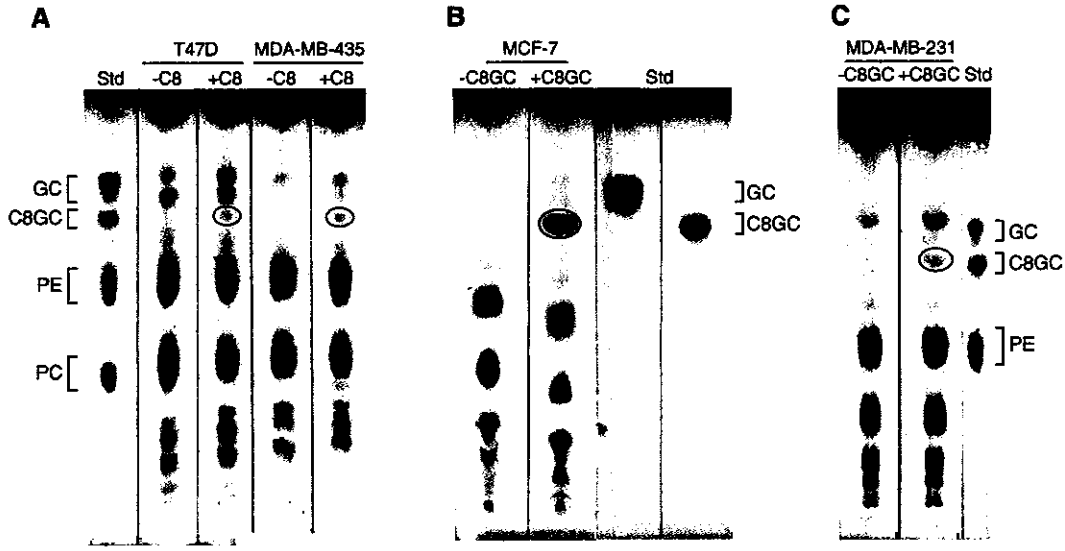

Fig. 2. Conversion of C8-ceramide to C8-GC and uptake of G8-GC when supplemented to breast cancer cells. (A) C8-ceramide conversion. Cells were grown in the absence and presence of C8-ceramide (5 μg/ml medium) for 72 h. (B, C) Uptake. Cells were grown in the absence and presence of C8-GC (10 μg/ml medium) for 48 h. Total lipids were extracted from washed cells and analyzed by TLC (200 μg total lipid/lane). Lipids were resolved using a solvent system containing chloroform/methanol/ammonium hydroxide (70:35:4, v/v), and visualization was by sulfuric acid char. Commercial standards (Std) used were GC, glucosylceramide (brain), C8-GC, phosphatidylethanolamine (PE), phosphatidylcholine (PC). In (A) the oval denotes C8-GC synthesized from C8-ceramide supplement. In (B, C) the oval denotes C8-GC that was found intracellularly after cells were incubated with C8-GC supplement.

and oleic acid exposure yielded no increase and a 1.4-fold increase, respectively, in MDR1 expression, over control.

3.2. Ceramide influence on MDR1 phenotype — chronic exposure

During testing of C8-ceramide influence on MDR1 mRNA levels in the various cell lines, we noted that MDA-MB-231 cells, after a 3-day exposure, were more sensitive to the cytotoxicity imparted by this ceramide analog compared to the other cell lines. We chose this cell line to study the influence of chronic exposure to C8-ceramide, the intent being to derive a ceramide tolerant cell line with enhanced capacity for ceramide clearance via glycosylation. This pathway would presumably enhance the levels of cellular C8-GC, and provide an alternate model for evaluating the impact of GC on the multidrug resistance phenotype. MDA-MB-231 cells were first grown with 2.5 μg/ml doses of C8-ceramide for two passages, after which the dose was increased to 5.0 μg/ml, with minimal cytotoxicity.

Prolonged growth of MDA-MB-231 cells in C8-ceramide-containing medium caused a robust increase in MDR1 mRNA levels. As measured by qPCR, after 5, 12, and 22 passages with C8-ceramide, MDR1 mRNA amounts increased by 22-, 44-, and 390-fold, compared to ceramide-naïve cells. Fig. 3A demonstrates, by gel electrophoresis of RT-PCR products, the dramatic increase in the level of MDR1 expression in MDA-MB-231/C8cer cells (at passage 22) compared to wild-type MDA-MB-231 cells. In order to determine if upregulation of MDR1 mRNA levels resulted in enhanced expression of P-gp protein, Western blot analyses were conducted. The results of Fig. 3B show that under the conditions employed, P-gp was undetectable in wild-type MDA-MB-231 cells; however, P-gp levels increased with increased C8-ceramide exposure time (passages 12 and 22). These data show that C8-ceramide exposure upregulates both MDR1 mRNA and protein. Prominent morphology changes also accompanied extended C8-ceramide exposure. Fig. 3C shows that MDA-MB-231/C8cer cells were rounder, larger, and tended to grow in islands consisting of clumped, multilayered cells compared to wild-type MDA-MB-231 cells which were spindle-shaped with long stellate cytoplasmic extensions.

Chemotherapy sensitivity status of MDA-MB-231/C8cer cells was evaluated and compared with wild-type MDA-MB-231 cells. Doxorubicin and paclitaxel, natural product chemotherapy drugs that are substrates for P-gp, were used. Firstly, MDA-MB-231/C8cer cells were not more resistant to C8-ceramide, when compared to MDA-MB-231 cells. The IC_{50} of C8-ceramide in MDA-MB-231 and MDA-MB-231/C8cer cells was 7.2 (3.1 μg/ml) and 7.6 μM (3.2 μg/ml), respectively. Because of the similar $1C_{50}$ values, we assessed GCS expression. Results from qPCR showed that chronic exposure of MDA-MB-231 cells to C8-ceramide (17 passages) downregulated GCS expression by approximately 12-fold, compared to wild-type MDA-MB-231 cells (based on number GCS gene copies/ copies β-actin). MDA-MB-231/C8cer cells were more resistant to anticancer drugs. As shown by dose-response/cell viability curves (Fig. 4), MDA-MB-231/C8cer cells were approximately 3- and 9-fold more resistant to doxorubicin and paclitaxel, respectively, compared to MDA-MB-231 cells. Computer-calculated IC_{50} values were 88 and 270 nM for doxorubicin in MDA-MB-231 and MDA-MB-231/C8cer cells, respectively, and 36 and 331 nM for paclitaxel in MDA-MB-231 and MDA-MB-231/C8cer cells, respectively.

In order to determine if the presence of P-gp in MDA-MB-231/C8cer cells correlated with higher efflux potential, we tested

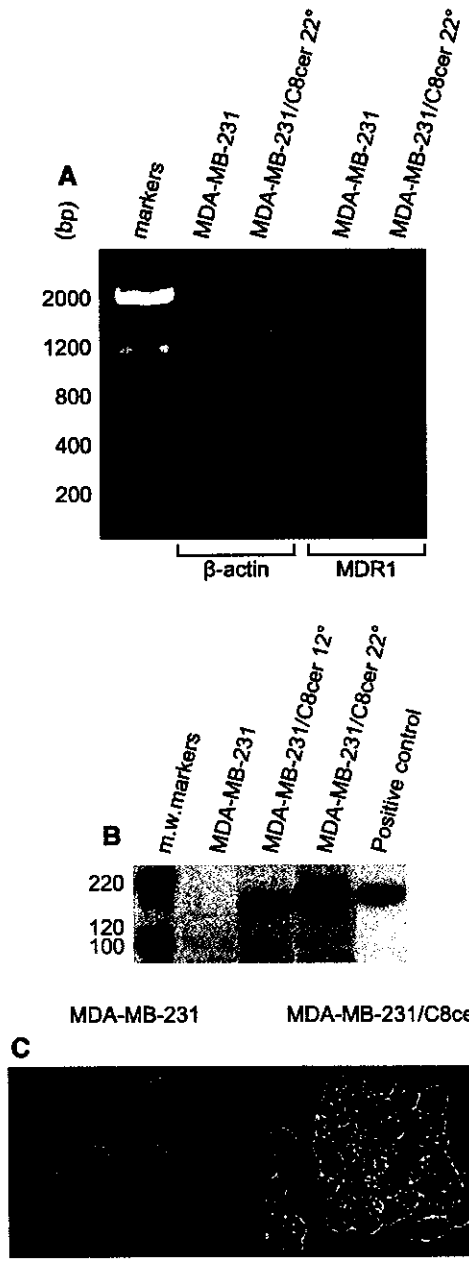

Fig. 3. The influence of chronic exposure to C8-ceramide on MDR1 mRNA, P-gp levels, and cell morphology in MDA-MB-231 cells. (A) MDR1 mRNA levels by RT-PCR in MDA-MB-231 cells and in high passage C8-ceramide cells (MDA-MB-231/C8cer, passage 22). Samples were subjected to RT-PCR analysis (0.5 μg RNA/tube) and products were resolved on 1% agarose gels. β-actin was employed as housekeeping gene. (B) P-gp levels by Western blot in MDA-MB-231 and in MDA-MB-231/C8cer cells at passages 12 and 22. Aliquots (100 μg cell protein) were electrophoresed for Western blot analysis of P-gp (C219 antibody). KB-Ch^R-8-5 (colchicine-resistant human epidermoid carcinoma) cell protein (50 μg) was used as a positive control for P-gp. For comparative purposes, 0.25 μg protein from MCF-7-AdrR cells was sufficient to detect P-gp in this highly drug-resistant cancer cell line (data not shown). (C) Cellular morphology. MDA-MB-231 cells, after first being exposed to 2.5 μg/ml C8-ceramide were grown continuously in the presence of 5 μg/ml C8-ceramide. MDA-MB-231/C8cer cells shown on right are from passage 19. Photomicrographs at 200× magnification.

the cells using rhodamine, a heterocyclic fluorescent compound that is a substrate for MDR1 protein. These experiments were conducted using cyclosporine A, a pump inhibitor, during rhodamine loading. This step eliminates efflux that occurs during uptake. Under these conditions, MDA-MB-231 and MDA-MB-231/C8cer cells took up approximately 1125 and 373 F.U., respectively, and retained 607 and 268 F.U., respectively, after the efflux period (Fig. 5). Further, the absence of cyclosporine A during uptake reduced rhodamine retained by only 22 and 21% in MDA-MB-231 and MDA-MB-231/C8cer cells, respectively. Therefore, the presence of P-gp in MDA-MB-231/C8cer cells did not impact extracellular effluxing of rhodamine. We further investigated the efflux potential of MDA-MB-231/C8cer cells using paclitaxel, a chemotherapy drug is a P-gp substrate. After 90 min, MDA-MB-231 and MDA-MB-231/C8cer cells took up 270±6 and 63±5 pmol [^{3}H]paclitaxel, respectively. Following a 90-min efflux period, MDA-MB-231 and MDA-MB-231/C8cer cells lost 120±5 (44%) and 28±2 (44%) pmol of [^{3}H]taxol, respectively.

3.3. Cellular metabolism of short-chain ceramides

In order to gain insight into the nature of the lipid causal in modifying MDR1 expression, we conducted in depth metabolism studies. The data in Table 1 show the influence of C8-ceramide on cellular ceramide synthesis from [^{3}H]palmitic acid in the breast cancer cell lines. Exposure to C8-ceramide enhanced synthesis of ^{3}H-ceramide in two cell lines, MDA-MB-231 and MCF-7, by 83 and 50%, respectively, whereas C8-ceramide supplements did not alter incorporation of radiolabeled palmitate into ceramide in T47D and MDA-MB-435 cells. We next assessed the capacity of the various cell lines to glycosylate C8-ceramide to C8-GC, by simultaneous incubation with C8-ceramide and [^{3}H]galactose. Using zonal profile analyses of the thin-layer chromatograms, the data of Fig. 6 demonstrate that each cell line has the capacity to glycosylate C8-ceramide, albeit to different degrees. In Fig. 6, the left-most peak corresponds to tritium migrating with commercial C8-GC standard and the right-most peak corresponds to lipid counts migrating with natural GC (glucocerebroside, Gaucher spleen). Of the total counts in monohexosylceramides, C8-GC accounted for 68, 26, 50, and 28% in MDA-MB-231, MDA-MB-435, MCF-7, and T47D, respectively. Notably, in some cell lines, the presence of C8-ceramide in the medium increased the synthesis of natural GC; for example, radiolabel in GC was increased by 25 and 255% in T47D and MCF-7 cells, respectively, (Fig. 6B, C), compared to cells in the absence of supplement.

To obtain a more detailed analysis of short-chain ceramide metabolism, including possible conversion to SPM and LacCer, we exposed cells to [1-^{14}C]C6-ceramide. The [^{14}C]C6-ceramide was diluted with unlabeled C6-ceramide to obtain a concentration in the medium that mimicked the C8-ceramide exposure level wherein MDR1 expression was enhanced (Fig. 1). The data of Fig. 7 confirm the capacity of the various breast cancer cell lines to glycosylate short-chain ceramides (see Fig. 6 for comparison), but more clearly demonstrate the high extent to which this conversion occurs. MDA-MB-231 cells converted 80% of the counts taken

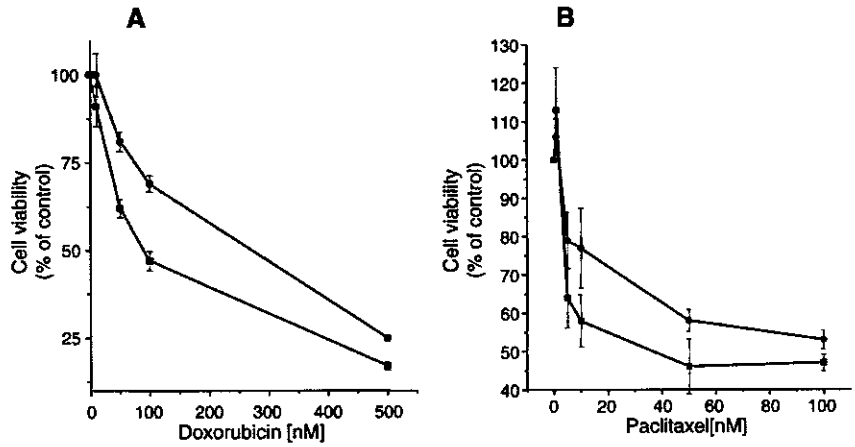

Fig. 4. Doxorubicin and paclitaxel sensitivity in MDA-MB-231 (■) and MDA-MB-231/C8cer cells (●). MDA-MB-231/C8cer cells (passage 17) were used in the chemosensitivity assays, (A) doxorubicin and (B) paclitaxel. C8-ceramide was not in the medium during the experiment. Cell viability was conducted as described in Materials and methods. Data are the mean±S.D. of six replicates. Experiments were repeated giving similar results.

up to [^{14}C]C6-GC in a 24 h period, and MDA-MB-435, MCF-7, and T47D cells had approximate conversion rates of 58, 62, and 58%, respectively. All cell lines synthesized [^{14}C]C6-LacCer, with MDA-MB-435 cells demonstrating the highest conversion rate, approximately 10% of total counts. All cell lines synthesized C6-SPM from [^{14}C]C6-ceramide, with counts ranging from 8 to 18% of total radioactivity. The presence of SPM was verified by TLC purification of the radioactivity migrating with commercial C6-SPM. This isolate was treated with SPMase (see Materials and methods). The radiolabeled lipid from the SPMase reaction, when assessed by TLC, migrated with commercial C6-ceramide. Metabolism of [1-^{14}C]C6-ceramide was also evaluated in MDA-MB-231/C8-cer cells (passage 17). As shown in Fig. 7, right panel, ceramide radioactivity accounted for >60% of the intracellular lipids, and GC counts, in contrast to MDA-MB-231 wild-type cells, amounted to only 20% of total. Therefore, MDA-MB-231/C8-cer cells, compared to wild-type, sluggishly convert [^{14}C]C6-ceramide to [^{14}C]C6-GC.

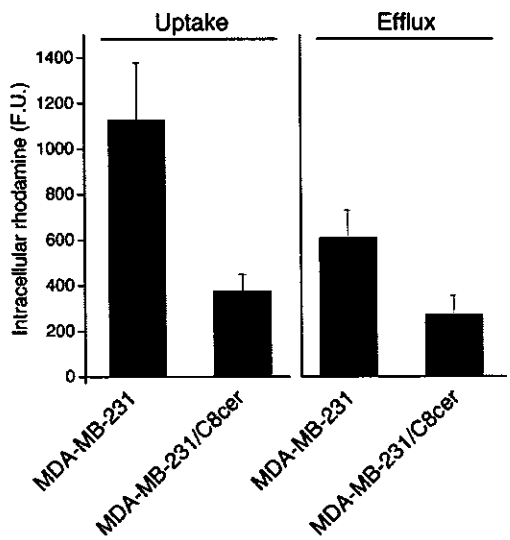

Fig. 5. Rhodamine uptake and efflux in MDA-MB-231 and MDA-MB-231/C8cer cells. The experiments were conducted as detailed in Materials and methods. Cyclosporine A was present during rhodamine uptake. F.U., fluorescence unites. Uptake refers to intracellular F.U. after 60 min rhodamine exposure. Efflux refers to intracellular F.U. remaining after 60 min reincubation.

3.4. MDA-MB-231/C8cer cell stability

In order to determine whether removal of C8-ceramide from the growth medium of MDA-MB-231/C8cer cells would cause the cells to revert, C8-ceramide was removed for 10 passages. Experiments were initiated using MDA-MB-231/C8cer cells at passage 17. Cellular morphology remained identical with that of MDA-MB-231/C8cer cells (see Fig. 3C) after C8-ceramide was removed for 10 passages. Further, the high levels of MDR1 mRNA did not decrease upon removal of C8-ceramide from the medium of MDA-MB-231/C8cer cells. The low GCS expression (mRNA) remained low in MDA-MB-231/C8cer cells cultured without C8-ceramide. Sensitivity to C8-ceramide was slightly enhanced when cells were cultured for 10 passages in the absence of ceramide supplement. For example, the IC_{50} values were 7.6 and 3.0 μM in MDA-MB-231/C8cer and MDA-MB-231/C8cer minus cells, respectively. Lastly metabolism of

Table 1
Influence of C8-ceramide on cellular ceramide synthesis from [^{3}H]palmitic acid

Cell line	^{3}H-Ceramide (cpm/100,000 cpm total lipid)	
	minus C8-cer	plus C8-cer
MDA-MB-231	347±25	635±19
T47D	1,470±178	1,478±122
MCF-7	1,142±51	1,722±94
MDA-MB-435	227±57	233±30

Cells (70% confluent) were incubated in 6-well plates without and with C8-ceramide (5 μg/ml medium) in culture medium containing 1.0 μCi/ml [^{3}H] palmitic acid for 24 h. Ceramide was resolved from total lipid extract by TLC.

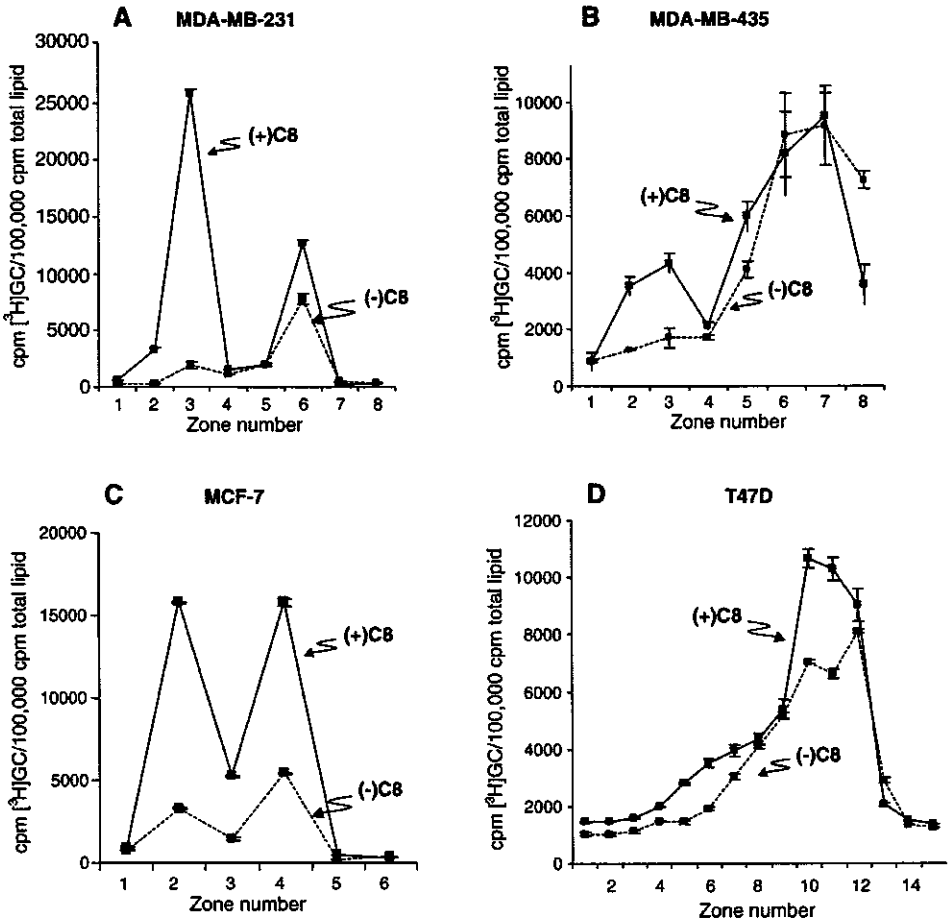

Fig. 6. Metabolism of and influence of C8-ceramide on GC formation in breast cancer cells. Cells in 6-well plates were grown in medium containing [^{3}H]galactose (10 μCi/ml 5% FBS medium) in the absence and presence of C8-ceramide (5 μg/ml) for 24 h. Cellular total lipid extracts were analyzed by TLC. Zonal analysis (incremental scraping) was done by LSC only on the GC area of the chromatogram. Results are the mean±S.D. from triplicate cultures.

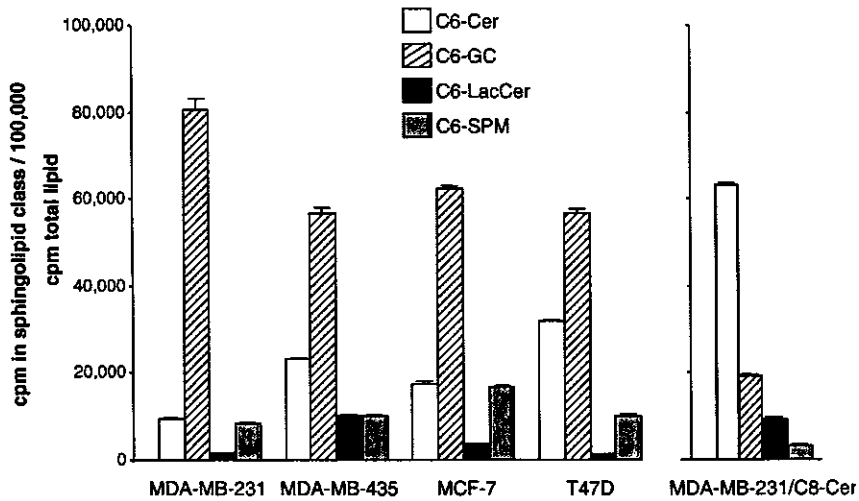

Fig. 7. Metabolism of [^{14}C]C6-ceramide by breast cancer cell lines. Cells in 6-well plates were grown with [^{14}C]C6-ceramide (500,000 cpm/ml, 5 μg/ml) for 24 h, after which total lipids were extracted and analyzed by TLC for radiolabeled free C6-ceramide, C6-GC, C6-SPM and C6-LacCer by LSC. For MDA-MB-231/C8cer cells (see right panel), the C8-ceramide was removed from the medium at seeding (6-well plates) and was also absent during the [^{14}C]C6-ceramide exposure period. Results are the mean±S.D. from triplicate cultures.

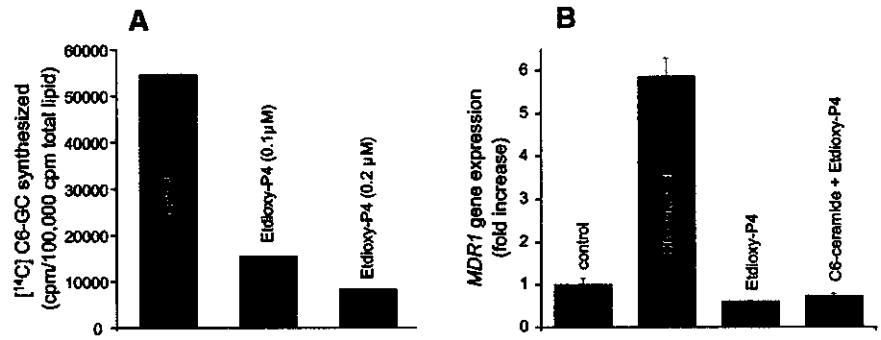

Fig. 8. Influence of GCS inhibition on ceramide-induced MDR1 upregulation in MDA-MB-435 cells. (A) Influence of ethylenedioxy-P4 (Etdioxy-P4) on cellular GC synthesis. Monolayer cultures (6-cm dishes) were incubated with [^{14}C]C6-ceramide mixed with unlabeled C6-ceramide (84,500 cpm/ml, 5 μg/ml) for 24 h in the absence or presence of ethylenedioxy-P4. [^{14}C]CG-GC was quantitated in the total lipid extract by TLC and LSC. (B) Influence of GCS inhibitor, ethylenedioxy-P4, on MDR1 expression. Cells in 10-cm dishes were exposed to the agents indicated for 24 h. Total RNA was extracted and MDR1 mRNA was measured by qPCR. C6-ceramide, 5.0 μg/ml; ethylenedioxy-P4, 0.2 μM. Fold increase was calculated as in Fig. 1.

[^{14}C]C6-ceramide by MDA-MB-231/C8cer cells after removal of supplement was not different from that depicted in Fig. 7, right panel, for MDA-MB-231/C8cer cells.

3.5. Blocking conversion of C6-ceramide to GC

We have shown that exposure of cells to C8-ceramide and C8-GC increases the expression of MDR1 (Fig. 1), and specifically with regard to ceramide metabolism, all cell lines examined robustly metabolized ceramide to GC (Fig. 7). Using MDA-MB-435 cells, which respond well to ceramide (Fig. 1A), we tested whether interfering with the conversion of ceramide to GC would impede upregulation of MDR1. The experiment depicted in Fig. 8 shows that the GCS inhibitor ethylenedioxy-P4 at low concentrations, effectively inhibits [^{14}C]C6-ceramide conversion to [^{14}C] C6-GC in MDA-MB-435 cells (Fig. 8A); for example, at a concentration of 0.2 μM, ethylenedioxy-P4 inhibited GC synthesis by nearly 90%. Fig. 8B demonstrates that whereas C6-ceramide exposure resulted in a near 6-fold enhancement of MDR1 expression, coadministration of ethylenedioxy-P4 and C6-ceramide completely blocked MDR1 upregulation. These data suggest that GC could participate in upregulating *MDR1* gene expression.

4. Discussion

We and others have shown that multidrug-resistant cancer cells, compared to drug-sensitive cells, contain high levels of GC [8,22,23]. Glucosylceramides and derivatives, found abundantly in nature, are involved in a myriad of processes including proliferation [24], oncogenic transformation [25,26], differentiation [27], and tumor metastasis [26,28]. In skin, GC production is responsible for normalized keratinocyte proliferation wherein upregulation of GCS protects against excess ceramide levels provoked by stress [29]. Other studies have demonstrated that LacCer synthase is upregulated in MDR1-overexpressing tumor cells [30] and that selection pressure for resistance to natural product chemotherapy selects for enhanced ceramide metabolism through GCS, in addition to upregulated P-gp expression [5]. These works draw a curious parallel between glycolipids and multidrug resistance caused by P-gp.

P-gp is expressed in some normal tissues such as kidney, pancreas, intestinal mucosa, adrenal gland, testis, and capillaries of brain. Cancers derived from these tissues may therefore be intrinsically drug-resistant. However, acquired drug resistance is common and occurs during chemotherapy in ovary, lung, bladder, and tumors of the breast. It is well known that anticancer agents, doxorubicin, vinblastine, etoposide, cytarabine, methotrexate, and paclitaxel for example, induce multidrug resistance through direct activation of the *MDR1* gene [31,32]. To date, ceramide has not been listed as a MDR1 activator. More recent studies have demonstrated transcriptional activation of the *MDR1* gene by UV irradiation [33] and by heat-shock transcription factor 1 [34]. A heat-shock independent induction of multidrug resistance by heat-shock factor 1 has also been described [35]. Although considerable progress has been made, the complexity of the ABC transporter family of proteins and their role in obstructing therapeutic goals necessitates that we improve our understanding of the mechanisms underlying their expression.

To our knowledge, the present work is the first showing that ceramide can lead to upregulated expression of *MDR1*. Upregulation of MDR1 was based on: (i) increased message as quantitated by both qPCR and gel electrophoresis, (ii) increased P-gp protein levels. The small increase in resistance to natural product anticancer agents (Fig. 4), while a characteristic of multidrug resistance, appears not to be the result of effluxing to the extracellular milieu, because uptake and efflux dynamics (Fig. 5) were not in line with drug pumping. The rhodamine efflux capacity of the P-gp-containing model (see Fig. 5) did not differ appreciably from wild-type MDA-MB-231 cells. Bates and Fojo and colleagues [36] have shown that although expression of the *MDR1* gene and gene product can be increased by various agents, this change is not necessarily associated with a decrease in cytotoxic drug accumulation, as was assessed by vinblastine uptake. Several other factors may account for low efflux capacity in MDA-MB-231/C8cer cells. Firstly, when compared to MCF-7-AdrR cells, MDA-MB-231/C8cer cells exhibited an approximate 800-fold lower amount of P-gp; this was based on Western blot analysis of P-gp

per unit total cell protein (unpublished data, investigators laboratory). Secondly, although MDA-MB-231/C8cer cells contain P-gp (see Fig. 3), whether it is plasma membrane-localized is not known, and plasma membrane localization is required for effluxing to the extracellular milieu. Wojtal et al. [37] have recently shown that trafficking of P-gp to apical canalicular plasma membrane requires GC.

Many studies have shown that P-gp is present in cancer cells and brain in the Golgi apparatus, rough ER, and the nuclear envelope [38–42]. Maraldi et al. [39] suggest, from work with MDR1 gene-transfected osteosarcoma, that in vitro drug resistance is largely dependant on expression of cell surface P-gp. Molinari et al. [40], in studying cultured tumor cells, show that cytoplasmic P-gp regulates intracellular drug traffic and thus impacts cellular drug targeting. Lastly, it is clear that MDA-MB-231/C8cer cells take up less rhodamine compared to wild-type (see Fig. 5), and less paclitaxel. Altered membrane lipid composition is known to influence cellular response to chemotherapy and the activity of membrane-bound enzymes, which in turn has been shown to impact uptake and sensitivity to anticancer agents [43–46]. These factors may underlie the physiochemical response of MDA-MB-231/C8cer cells to rhodamine, paclitaxel, and doxorubicin. However, it is important to note that the small increase in resistance observed is more in line with what is seen clinically, as many of the established multidrug resistant cancer cell lines, MCF-7-AdrR for example, demonstrate extreme drug resistance and have high P-gp levels that are not clinically relevant [47]. For example, cells selected in culture overexpress P-gp as much as 100-fold, whereas human cancers overexpress P-gp 2–5-fold [48], which is adequate to confer resistance [49,50].

In this study, some cell lines responded to short-chain GC supplements whereas other cell lines responded to short-chain ceramides (C8 and C6). These disparate responses may be linked to distinct physicochemical behavior of the analogs as well as distinct neighborhood topology. It is possible that C8-GC is poorly active in T47D and MDA-MB-435 cells because the GC must be synthesized in situ to be active in MDR1 modulation. GC is synthesized on the cytoplasmic surface of early Golgi and converted to LacCer, in the lumen of the Golgi presumably after being "flipped in" [51,52]. Along these lines, Tepper et al. [53] have noted specific differences in cellular ceramide metabolism related to topological segregation, in particular with GCS access. It is possible that ceramide, because of the proximity of the enzymatic machinery, is physiologically favored, after conversion to GC, as a substrate of MDR1 modulation.

The metabolic studies using [^{14}C]C6-ceramide were conducted using concentrations that mimicked the MDR1 induction experiments (see Fig. 1). This was done to challenge the cells and determine how GCS, would metabolize high concentrations of ceramide. All cell lines readily converted ceramide to GC (see Fig. 7); SPM and LacCer were generated, but to a lesser extent. It is well known that P-gp can serve as a flipase for glycosphingolipids including GC [54]. Other ABC transporters also have broad specificity for lipid translocation [15,55,56]. Hence, supplying the cell with an abundance of substrate, in this case GC, could induce enzyme upregulation, in this case, P-gp. Our experiments show that introduction of a GCS inhibitor to ceramide-supplemented cells eliminated MDR1 increases (see Fig. 8), suggesting that GC influences MDR1 expression. However, at this point it is not possible to determine which lipid is responsible for MDR1 upregulation. The association of GCS with MDR1 was recently demonstrated by experiments showing that inhibition of GCS severely limits the expression of MDR1 and its product, P-gp (12). It should be noted, however, that increased MDR1 expression is not always accompanied by enhanced conversion of ceramide to GC, as gleaned from Fig. 7, right panel. Thus, complex mechanisms likely account for MDR1 upregulation.

In similar studies on the metabolism of sphingolipid analogs, Radin, Shayman, and colleagues [57] showed, in Madin–Darby canine kidney cells, that supplementation with C8-GC and C8-ceramide produced increases in natural GC, ceramide, and free sphingosine, and that radiolabeled C8-ceramide yielded radiolabeled GC and sphingomyelin; hydrolysis of lipids was also noted. Ogretmen et al. [58] have shown that short-chain ceramide can be enzymatically cleaved and the backbone reacylated to form long-chain ceramide counterpart. We do not know whether these ceramides participate in the MDR1 response; however, results in Table 1 suggest little cleavage of C8-ceramide occurred.

The role of lipids in multidrug resistance has been the subject of a recent review [59], and although lipids can impact drug transport and membrane fluidity, and perhaps P-gp's integrity and stability in the plasma membrane, little is known about the influence of lipids on *MDR1* gene expression. The relevance of sphingolipids to transcriptional regulation of ABC drug transporters [60,61] could be major, and with ceramide and metabolites, the intracellular signaling pathways that participate, including protein kinase C [62], are vast. Perhaps ceramide's role as a messenger of cytotoxic response to chemotherapy is linked to the multidrug resistance pathway. We hope these results inspire new studies in the area of lipids and multidrug resistance.

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Glucosylceramide Synthase Blockade Down-Regulates P-Glycoprotein and Resensitizes Multidrug-Resistant Breast Cancer Cells to Anticancer Drugs

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Abstract

Overexpression of glucosylceramide synthase (GCS), a pivotal enzyme in glycolipid biosynthesis, contributes to cancer cell resistance to chemotherapy. We previously showed that transfection of doxorubicin-resistant MCF-7-AdrR cells with GCS antisense restored cell sensitivity to doxorubicin and greatly enhanced sensitivity to vinblastine and paclitaxel. In that study, doxorubicin promoted generation of ceramide in MCF-7-AdrR/GCS antisense cells; the present study implicates factors in addition to ceramide that augment sensitivity to chemotherapy. Although GCS antisense cells showed enhanced ceramide formation compared with MCF-7-AdrR when challenged with paclitaxel, GCS antisense cells also showed a 10-fold increase in levels of intracellular drug (paclitaxel and vinblastine). In addition, transfected cells had dramatically decreased expression (80%) of P-glycoprotein and a 4-fold decrease in the level of cellular gangliosides. Chemical inhibition of GCS produced the same effects as antisense transfection: exposure of MCF-7-AdrR cells to the GCS inhibitor 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP, 5.0 μmol/L, 4 days) decreased ganglioside levels, restored sensitivity to vinblastine, enhanced vinblastine uptake 3-fold, and diminished expression of MDR1 by 58%, compared with untreated controls. A similar effect was shown in vinblastin-resistant KB-V0.01 cells; after 7 days with PPMP (10 μmol/L), MDR1 expression fell by 84% and P-glycoprotein protein levels decreased by 50%. MCF-7-AdrR cells treated with small interfering RNAs to specifically block GCS also showed a dramatic decrease in MDR1 expression. This work shows that limiting GCS activity down-regulates the expression of MDR1, a phenomenon that may drive the chemosensitization associated with blocking ceramide metabolism. The data suggest that lipids play a role in the expression of multidrug resistance. (Cancer Res 2005; 65(9): 3861-7)

Introduction

Development of resistance to chemotherapeutic agents is a major concern in cancer patients. Resistance to chemotherapy is associated with myriad mechanisms that decrease drug cytotoxicity. Two members of the large family of ABC transporters confer multidrug resistance (MDR) in human cancer cells: P-glycoprotein and multidrug resistance protein (MRP). P-glycoprotein, a membrane-resident glycoprotein encoded by the *MDR1* gene, decreases the intracellular concentration of anticancer agents by acting as a drug efflux pump (1, 2). P-glycoprotein exports many types of chemotherapy drugs, including *Vinca* alkaloids, anthracyclines, paclitaxel, actinomycin D, and epipodophyllotoxins. Like P-glycoprotein, MRP is a transport protein (3); however, the transport of unconjugated chemotherapeutic agents by MRP seems to require glutathione. MDR in tumors can also be caused by overexpression of proteins such as antiapoptotic proteins belonging to the Bcl-2 family (4, 5) and by loss of tumor suppressor protein p53 (6, 7). Others factors responsible for chemotherapy resistance include reduction of topoisomerase II activity (8), modification of glutathione *S*-transferase activity (9), and up-regulation of rafts and caveolae, which are glycosphingolipid-enriched constituents of microdomains (10).

Glucosylceramide synthase (GCS) catalyzes the first glycosylation step in the biosynthesis of glycosphingolipids (11, 12). This central enzyme of ceramide metabolism has also been implicated in MDR (13). Glycosphingolipids, including glucosylceramide, lactosylceramide, and gangliosides, play an essential role in cell development, cell death, tumor progression, and pathogen/host interaction (13, 14). In addition, membrane gangliosides can decrease the sensitivity of human melanoma cells to ionizing radiation (15). In that study, radioresistant melanoma cells were made radiosensitive by exposure to either fumonisin B_1, which blocks ganglioside biosynthesis at the juncture of ceramide synthase, or *Vibrio* cholerae neuraminidase, which cleaves cell surface gangliosides. Conversely, adding bovine brain GM1 to radiosensitive melanoma cells conferred radioresistance (15). Targeting glycolipid metabolism has proven useful in altering chemotherapy responses in numerous human tumor cell lines (13, 16–18).

In previous studies, we increased the level of MDR by transfecting doxorubicin-resistant human breast cancer cells (MCF-7-AdrR) with GCS, and we enhanced cellular sensitivity to anthracyclines, *Vinca* alkaloids, and taxanes by transfecting MCF-7-AdrR cells with GCS antisense (16, 19). Although doxorubicin treatment of GCS antisense transfectants increased intracellular levels of ceramide (16), which is a second messenger of apoptosis, the extremely high sensitivity of MCF-7-AdrR/GCS antisense cells to *Vinca* alkaloids and taxanes suggested the participation of mechanisms other than ceramide signaling in cellular responses. We have observed equivalent intracellular levels of rhodamine-123 in MCF-7-AdrR/GCS antisense cells and in rhodamine-123–exposed MCF-7 parental cells, which indicates that GCS antisense transfection reverts drug retention in MCF-7-AdrR cells on a par with the drug-sensitive phenotype (16, 19). Because rhodamine-123 is a substrate for P-glycoprotein, we began to investigate the influence of glycolipid metabolism on function and expression of MDR1 and P-glycoprotein. The present study shows that MDR1 and

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P-glycoprotein expression can be down-regulated by GCS antisense transfection or chemical inhibition of GCS. We suggest that this avenue contributes to antisense GCS chemosensitization of drug-resistant cells. Overall, our work shows that lipids play a role in multidrug resistance and that targeting glycolipid biosynthesis could be a promising approach for enhancing chemotherapy.

Materials and Methods

Cell cultures. The MCF-7-AdrR human breast adenocarcinoma cell line, which is resistant to doxorubicin (20), was kindly provided by Dr. Kenneth Cowan (UNMC Eppley Cancer Center, Omaha, NE) and Dr. Merrill Goldsmith (NIH, Bethesda, MD). MCF-7-AdrR cells were maintained in RPMI 1640 (Invitrogen, Chicago, IL) containing 10% (v/v) fetal bovine serum (FBS; HyClone, Logan, UT), 100 units/mL penicillin, 100 μg/mL streptomycin, and 584 mg/L L-glutamine. Cells were cultured in a humidified, 5% CO_2 atmosphere tissue culture incubator and subcultured weekly using trypsin-EDTA (0.05%, 0.53 mmol/L) solution. MCF-7-AdrR cells transfected with GCS antisense (MCF-7-AdrR/GCS antisense) were cultured in RPMI 1640 containing the above components and G418 (400 μg/mL; ref. 16).

The KB-V0.01 multidrug-resistant human epidermoid carcinoma cell line (21) was grown in high glucose (4.5 g/L) DMEM with 10% fetal bovine serum and vinblastine (10 ng/mL). Cells were a gift from Dr. Michael Gottesman (National Cancer Institute, Bethesda, MD).

Cytotoxicity assays. Assays were done as described previously (13). Briefly, cells were seeded in 96-well plates (5,000 cells per well) in 0.1 mL RPMI 1640 containing 10% FBS and cultured at 37°C for 24 hours before addition of vinblastine sulfate or paclitaxel (Taxol), both of which were obtained from Sigma (St. Louis, MO). Drugs were added in FBS-free medium (0.1 mL), and cells were grown at 37°C for the indicated periods. Drug cytotoxicity was determined by the CellTiter 96 AQueous One Solution cell proliferation assay (Promega, Madison, WI). Absorbance at 490 nm was measured by a fluorescent reader (Microplate FL600, Bio-Tek, Winooski, VT).

Analysis of lipids. Analysis was done as described previously (13, 22). Briefly, for ceramide, sphingomyelin, phospholipid, and neutral lipid analysis, cells were seeded 6-well plates (60,000 cells per well) in 10% FBS RPMI 1640. After 24 hours, medium was replaced with 5% FBS medium containing [^{3}H]palmitic acid (1.0 μCi/mL culture medium; DuPont/NEN, Boston, MA) for 24 hours. Total lipids were extracted as described (22), and the resulting organic lower phase was withdrawn and evaporated under a stream of nitrogen. Lipids were resuspended in 0.1 mL chloroform/methanol (2:1, v/v), and resolution was by TLC using solvent systems described (23). Commercial lipid standards (Avanti Polar Lipids, Alabaster, AL) were cochromatographed. After development, lipids were visualized by iodine vapor staining, and the area of interest was scraped into 0.5 mL water. EcoLume scintillation fluid (4.5 mL; ICN, Costa Mesa, CA) was added, samples were mixed, and radioactivity was quantitated by liquid scintillation spectrometry. For ceramide quantitation, cells were grown in 15-cm dishes, and total lipids were extracted and analyzed by TLC using a solvent system containing chloroform/acetic acid (90:10, v/v). Approximately 880 μg of lipid were loaded per lane. After development, TLC plates were sprayed with conc. sulfuric acid, and heated in an oven (30 minutes, 180°C). The ceramide chars were quantitated by scanning densitometry, using a ceramide standard curve (1.0-6.0 μg). Silica Gel G prescored TLC plates were purchased from Analtech (Newark, DE). Before the above procedure, plates were acid washed by running in methanol/HCl (90:10, v/v), and thoroughly dried before use.

Western blot for P-glycoprotein. Confluent monolayers of MCF-7-AdrR and MCF-7-AdrR/GCS antisense cells were rinsed, harvested in PBS, and lysed in a PBS buffer containing 10% glycerol, 1% Triton X-100, 1.0 mmol/L Na_3VO_4, 10 mmol/L β-glycerophosphate, 50 mmol/L NaF, 0.1 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, and 10 μg/mL aprotinin for 30 minutes on ice. The mixture was centrifuged at 11,000 × *g* for 15 minutes at 4°C. Equal aliquots of protein (25 μg) were resolved using 4% to 20% gradient SDS-PAGE (Invitrogen, Chicago, IL). The transferred nitrocellulose blot was blocked with 5% fat-free milk powder in PBS containing 0.1% Tween 20, at room temperature for 1 hour. The membrane was immunoblotted with 0.7 μg/mL of C219 murine monoclonal antibody against human P-glycoprotein (Calbiochem, Pasadena, CA) in the same blocking solution. Detection was done using enhanced chemoluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

RNA analysis. Total RNA was isolated using the RNeasy Protect Mini Kit from Qiagen, Inc. (Los Angeles, CA). MDR1 reverse transcription-PCR (RT-PCR) was carried out by a one-step method (SuperScript One-Step RT-PCR with Platinum Taq; Invitrogen, Chicago, IL). Total RNA (25 ng) was added to buffer containing 0.2 mM deoxynucleotide triphosphate, 1.2 mmol/L $MgSO_4$, 1.0 μL SuperScript II RT/Platinum Taq mix (containing reverse transcriptase and platinum Taq DNA polymerase), 0.2 μg of MDR1 upstream primer 5′-CCATCATTGCAATAGCAGG-3′, and 0.2 μg of MDR1 downstream primer 5′-GAGCATACATATGTTCAAACTTC-3′. RT-PCR, in a total volume of 50 μL, was done for 35 cycles in a thermocycler (Eppendorf Scientific, Westbury, NY); each cycle comprised denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 1 minute. RT-PCR products were analyzed by 1% agarose gel electrophoresis stained with ethidium bromide. *β-Actin* primer (Stratagene, Cedar Creek, TX) was used as a housekeeping gene.

Real-time PCR. A real-time quantitative PCR analysis was done using the Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Primers and probe sequences for MDR1 were chosen as follows: MDR1 forward 5′-GGTTTATAGTAGGATTTACACGTGGTTG-3′, MDR1 reverse 5′-AAGATAGTATCTTTGCCCAGACAGC-3′, and MDR1 probe 5′ FAM CTAACCCTTGTGATTTTGGCCATCAGTCC Tamra 3′. Human β-actin was used as endogenous control. Both assays used the SuperScript III Platinium one-step quantitative RT-PCR system (Invitrogen, Chicago, IL).

Glucosylceramide synthase gene silencing by small interfering RNA. The small interfering (siRNA) sequence targeting human GCS was selected using the BLOCK-IT RNAi Designer program; reagents were synthesized by Invitrogen (Carlsbad, CA). The siRNA duplex with the following sense and antisense sequences was used: 5′-CCAGGAUAUGAAGUUGCAA (sense) and 5′-UUGCAACUUCAUAUCCUGG (antisense). Established protocols were followed (24, 25). Briefly, siRNA was introduced into cells using LipofectAMINE 2000 in serum-free medium for 4 hours. FBS was added, and after 48 hours, total RNA was extracted and used to examine GCS and MDR1 mRNA levels. LipofectAMINE 2000 alone and expression of *β-actin* were used as controls.

Purification and analysis of gangliosides. Cells harvested in PBS were homogenized in 6 mL chloroform/methanol (1:1, v/v); the mixture remained overnight at room temperature. After centrifugation, the supernatant was dried and the lipid residue was taken up in chloroform/methanol (1:1) and centrifuged to remove all solid particles. Addition of PBS in a volume ratio of 1:1:0.7 (chloroform/methanol/PBS) separated the organic phase from the ganglioside-containing aqueous phase, as previously described (26). After thorough vortex mixing, the tube was centrifuged, and the upper phase containing gangliosides was withdrawn. Partitioning was repeated twice, each time by adding methanol/PBS (1:0.7, v/v) to the lower phase, followed by centrifugation. The upper phases were pooled, and gangliosides were recovered from the aqueous solution by column chromatography on C18-bonded silica gel. Ganglioside profiles were determined by high-performance TLC on Silica Gel 60 plates (Merck, Darmstadt, Germany) developed in chloroform/methanol/0.2% aqueous calcium chloride (55:45:10, v/v/v) and sprayed with resorcinol-HCl reagent. Ganglioside sialic acid content was determined by the periodate-resorcinol method (27).

[^{3}H]Paclitaxel and [^{3}H]vinblastine uptake studies. Cells were seeded into 12-well plates at 100,000 cells per well in 1.0 mL of complete medium. After 24 hours at 37°C, the medium was removed; cells were rinsed with PBS and incubated for 10 to 90 minutes with 0.5 mL of 5% FBS RPMI 1640 containing 500 nmol/L paclitaxel plus 0.25 μCi [^{3}H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 μCi [^{3}H]vinblastine (Moravek biochemical). After removal of culture medium, cells were washed twice with 5% FBS RPMI 1640 and lysed with 0.2 mL of 5% FBS RPMI 1640 containing 0.02% SDS. Intracellular radioactivity was measured by liquid scintillation counting.

MDR1 induction by glycolipids. KB-V0.01 cells were seeded into 6.0-cm dishes in complete medium. After 24 hours, medium was removed and cells were incubated in 5% FBS DMEM medium containing either 30 μg/mL C8 β-D-glucosylceramide (Avanti Polar Lipids, Alabaster, AL), 0.5 μmol/L doxorubicin (LKT Laboratories, St Paul, MN) as positive control, or 10 μg/mL palmitic acid (Sigma) as negative control. Cells were treated for 48 hours, and RNA was analyzed by real-time RT-PCR.

Chemical inhibition of glucosylceramide synthase. D,L-*Threo*-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) was from Biomol Research Laboratories (Plymouth Meeting, PA) and D,L-*erythro*-PPMP and D-*threo*-PPMP were from Matreya (Pleasant Gap, PA). These reagents were used as described in the figure legends.

Statistical analyses. Student's *t* test was used for statistical analysis.

Results

Previously, we showed that GCS antisense transfection of multidrug-resistant MCF-7-AdrR cells enhanced cell sensitivity to doxorubicin, vinblastine, and paclitaxel (19). A doxorubicin-induced increase in ceramide levels and caspase activity is in keeping with ceramide-mediated cytotoxic responses to chemotherapy (16), but it is not clear whether ceramide is the only factor involved in the significantly (>100-fold) increased sensitivity of GCS antisense–transfected cells to *Vinca* alkaloids and paclitaxel.

Initial studies on ceramide production measured with [^{3}H]palmitate showed that similar levels of tritiated ceramide were formed in both MCF-7-AdrR and MCF-7-AdrR/GCS antisense cells in response to vinblastine and paclitaxel challenge (Fig. 1*A*). Using mass analysis, however, we found that ceramide production in response to paclitaxel (1.0 μmol/L) was enhanced 3-fold in MCF-7-AdrR/GCS antisense cells compared with parental MCF-7-AdrR cells after 24 hours of treatment (Fig. 1*B*). To further assess the influence of GCS antisense transfection on cell response to chemotherapy, we next measured uptake and efflux variables of vinblastine and paclitaxel. P-glycoprotein–mediated drug efflux is the most widely characterized drug resistance mechanism in cancer cells (28), and it is highly expressed in MCF-7-AdrR cells (13). We previously reported that levels of rhodamine-123, a substrate of P-glycoprotein, were ~5-fold higher in MCF-7-AdrR/GCS antisense compared with MCF-7-AdrR cells (19). This suggests that GCS antisense transfection alters drug uptake and/or retention. Experiments with chemotherapy drugs showed that after 60 minutes, intracellular levels of vinblastine were 12-fold greater in MCF-7-AdrR/GCS antisense compared with MCF-7-AdrR cells (Fig. 2). Similarly, uptake of paclitaxel increased 8.6-fold in GCS antisense transfectants compared with MCF-7-AdrR cells (Fig. 2).

Because of the pronounced differences in drug levels in the two cell lines, we assessed P-glycoprotein expression, by mRNA and protein. As shown in Fig. 3, the level of MDR1 mRNA, evaluated by reverse transcription-PCR (RT-PCR), was dramatically lower in MCF-7-AdrR/GCS antisense compared with MCF-7-AdrR cells. We confirmed this by Western blot; whereas MCF-7-AdrR cells contained characteristically elevated levels of P-glycoprotein, MCF-7-AdrR/GCS antisense cells were nearly devoid of P-glycoprotein. These data suggest that high drug levels attainable in MCF-7-AdrR/GCS antisense cells are a consequence of the dramatic decrease (~80%) in P-glycoprotein expression. Thus, the stable 30% decrease in GCS activity of MCF-7-AdrR/GCS antisense cells (16) seems to have an important influence on intracellular drug levels and on P-glycoprotein expression. For this reason, we investigated whether partial inhibition of GCS would cause other alterations, in particular, in membrane lipid composition.

Steady-state [^{3}H]palmitic acid radiolabeling (24 hours) of MCF-7-AdrR/GCS antisense and MCF-7-AdrR cells showed, in the former, a 30% decrease in sphingomyelin levels and a 44% decrease in the level of phosphatidylinositols (data not shown).

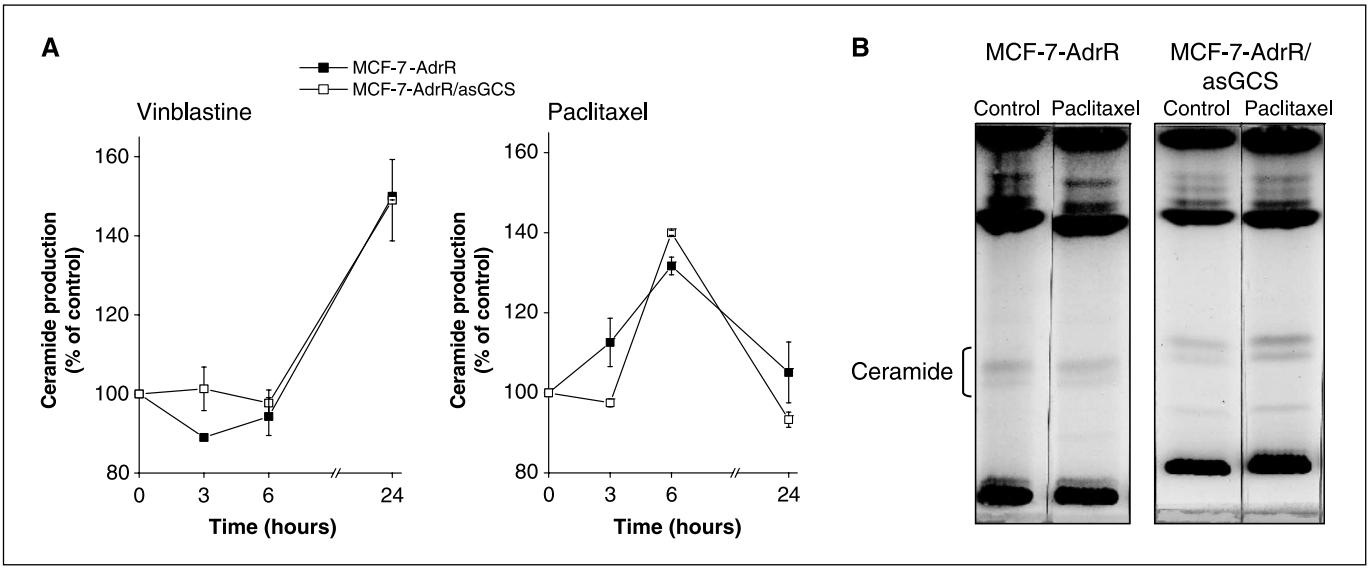

Figure 1. Influence of chemotherapy on ceramide formation in MCF-7-AdrR and MCF-7-AdrR/GCS antisense (*asGCS*) cells. *A,* assays using radiolabeling. Cells were treated with vinblastine (0.5 μmol/L) or paclitaxel (1.0 μmol/L) for the times shown in medium containing [^{3}H]palmitic acid. Tritiated ceramide was evaluated in the lipid extract by TLC and liquid scintillation spectroscopy as detailed in Materials and Methods. For comparison purposes, ceramide counts in MCF-7-AdrR control (no drug) were 11,890 ± 3,732 cpm/500,000 cpm total lipid and 11,084 ± 2,948 cpm/500,000 cpm total lipid in control (no drug) MCF-7-AdrR/GCS antisense cells. *Points,* mean from three experiments; *bars,* ±SD. *B,* ceramide assay using mass analysis. Cells were treated ± paclitaxel (1.0 μmol/L) for 24 hours and ceramide was evaluated by TLC sulfuric acid charring and photodensitometry as detailed in Materials and Methods. Ceramide mass in both MCF-7-AdrR control and MCF-7-AdrR paclitaxel-treated cells was 1.6 μg. Ceramide mass in MCF-7-AdrR/GCS antisense control and MCF-7-AdrR/GCS antisense paclitaxel treated was 1.0 and 3.25 μg, respectively. Total lipid (880 μg) was loaded in each lane.

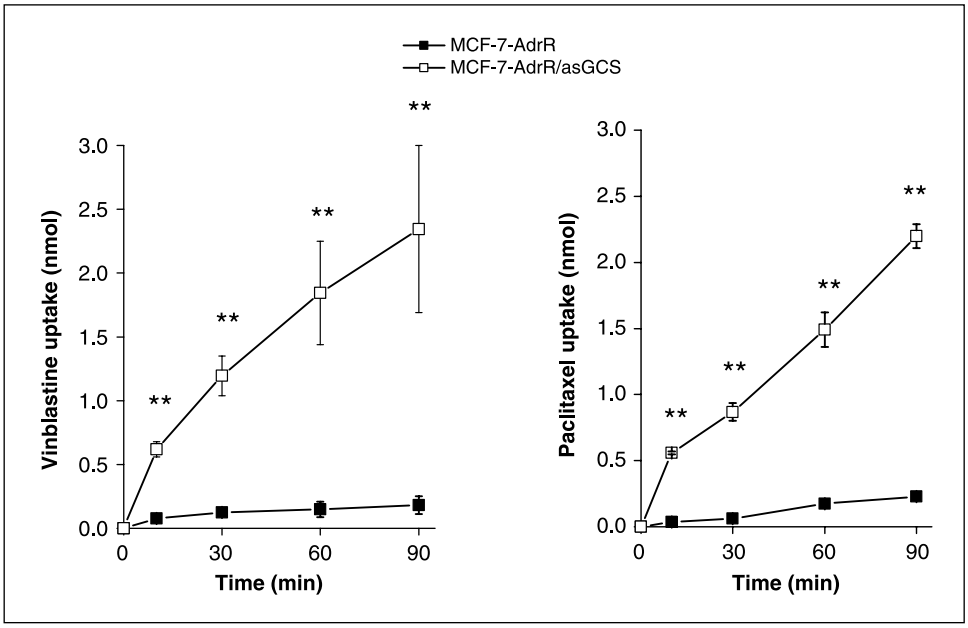

Figure 2. Uptake of vinblastine and paclitaxel in MCF-7-AdrR and MCF-AdrR/GCS antisense (*asGCS*) cells. *Points,* mean from two independent experiments; *bars,* ±SD. **, $P < 0.01$.

There were no significant differences in cholesterol esters or other glycerophospholipids between transfected and parent cells. Because GCS is pivotal in the genesis of cerebrosides and gangliosides, we also looked for changes in glycosphingolipid content. Although MCF-7-AdrR and MCF-7-AdrR/GCS antisense cells expressed a similar ganglioside pattern (GM3, GM2, GD3, and GD1a; data not shown), sialic acid assay showed that the level of gangliosides was 4-fold lower in GCS antisense transfected cells (data not shown).

To determine whether depletion of gangliosides and reduced expression of P-glycoprotein were strictly a consequence of GCS down-regulation by antisense transfection, we evaluated the influence of D,L-*threo*-PPMP, a chemical inhibitor of GCS (29–31), on ganglioside synthesis and P-glycoprotein expression in MCF-7-AdrR cells. A 4-day exposure to D,L-*threo*-PPMP produced a 34% decrease in ganglioside levels in MCF-7-AdrR cells (Fig. 4*A*). Moreover, PPMP greatly diminished the expression of MDR1 in MCF-7-AdrR cells, with shown stereospecificity (Fig. 4*B*). Unlike D,L-*threo*-PPMP, D,L-*erythro*-PPMP is not a GCS inhibitor (32), and this stereoisomer had no influence on MDR1 expression. Real-time RT-PCR showed that MDR1 expression in MCF-7-AdrR cells treated with D,L-*threo*-PPMP and D,L-*erythro*-PPMP was reduced by 58% and 12%, respectively, compared with untreated MCF-7-AdrR cells (Fig. 4*B*). To reinforce the results obtained with PPMP and to confirm that changes in MCF-7-AdrR/GCS antisense cellular MDR1 expression were not due to clonal artifacts, we used GCS siRNA to treat MCF-7-AdrR cells. As shown in Fig. 4*C*, after 48 hours, both GCS and MDR1 mRNA were dramatically decreased by GCS siRNA compared with LipofectAMINE only controls. The siRNA had no effect on expression levels of β-actin.

Whether chemical lowering of MDR1 expression affects cellular response to chemotherapy was next evaluated. Treatment of MCF-7-AdrR cells for 4 days with D,L-*threo*-PPMP enhanced vinblastine uptake by ~3-fold at 30 and 60 minutes (Fig. 4*D*), and as illustrated in Fig. 4*E*, vinblastine cytotoxicity, even at low concentrations (0.1 μmol/L), was enhanced ~60% in cells that had been cultured with D,L-*threo*-PPMP. Thus, like GCS antisense transfection, inhibition of GCS by chemical means reversed resistance of MCF-7-AdrR cells to vinblastine. To examine the generality of this response, we used KB-V0.01 cells, a head/neck multidrug-resistant epidermoid carcinoma cell line that expresses both GCS and MDR1 (33). As shown in Fig. 5*A*, MDR1 expression in KB-V0.01 cells was lowered 70% by D-*threo*-PPMP (10 μmol/L) and 38% by D,L-*threo*-PPMP (15 μmol/L). Therefore, the D-*threo* isomer is the most effective inhibitor of GCS compared with the racemic mixture. KB-V0.01 cell treatment with D-*threo*-PPMP for a prolonged period (7 days) induced a dramatic decrease (84%) in MDR1 mRNA levels (Fig. 5*B*). Moreover, P-glycoprotein protein levels in these cells diminished by 50%, compared with the untreated control (Fig. 5*C*). We next investigated the effect of glycolipid supplementation on MDR1 expression. Growth of KB-V0.01 cells with cell-permeable C8-glucosylceramide (30 μg/mL) elicited a 2-fold increase in MDR1 mRNA levels (Fig. 6), a response nearly comparable to the influence of Adriamycin (0.5 μmol/L) on MDR1 expression (Fig. 6). Palmitic acid, used as a lipid control, had no influence on MDR1 expression.

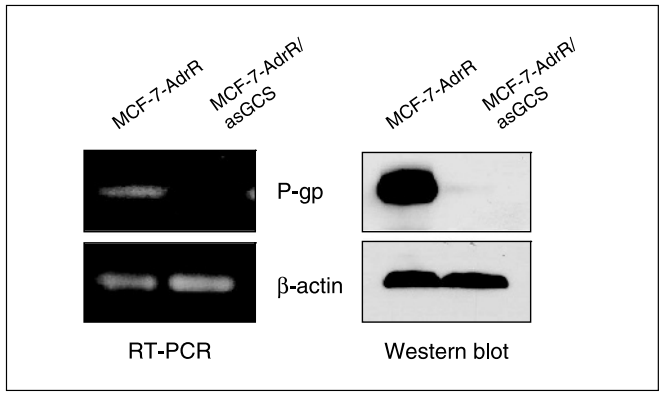

Figure 3. P-glycoprotein (*P-gp*) expression in MCF-7-AdrR and MCF-7-AdrR/GCS antisense (*asGCS*) cells. MCF-7-AdrR and MCF-7-AdrR/GCS antisense cells were grown to 70% confluence, and RNA and protein were extracted and used for RT-PCR and Western blot analysis. The C219 murine monoclonal antibody against human P-glycoprotein was used for the Western blot.

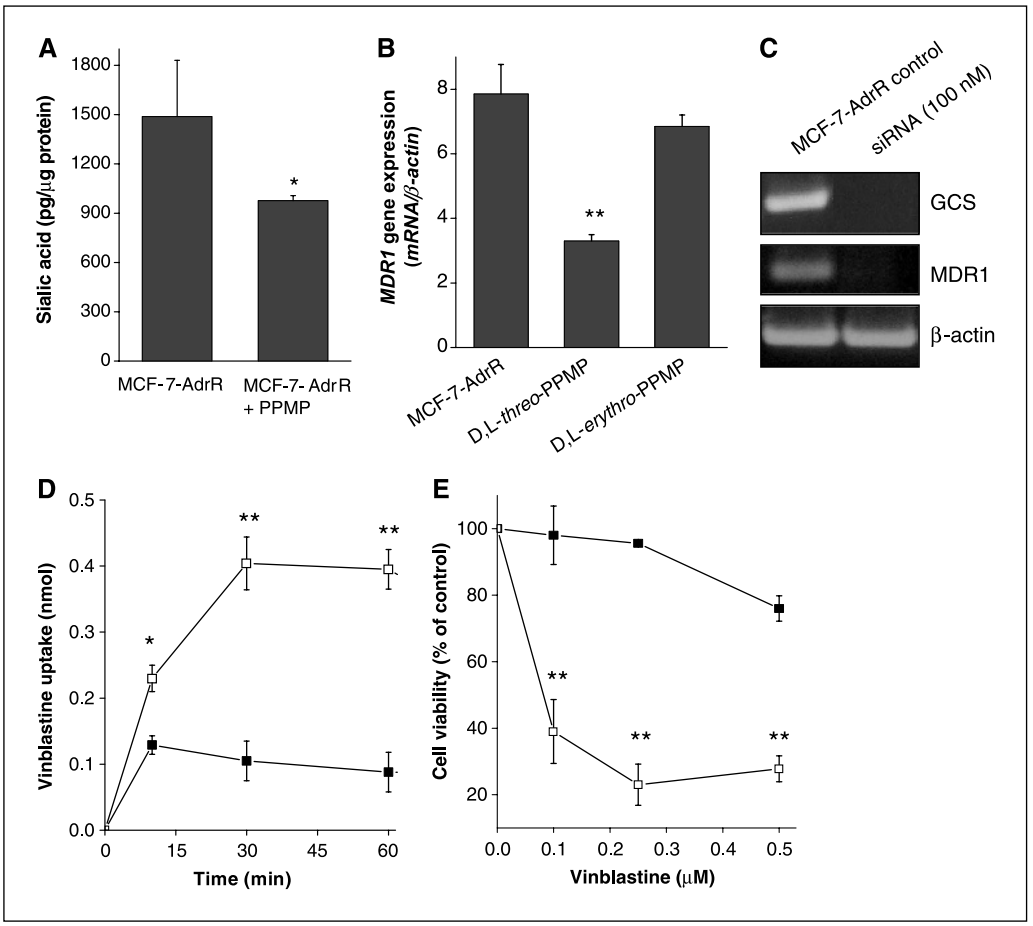

Figure 4. Influence of GCS blockade via PPMP and siRNA on ganglioside levels, MDR1 expression, and drug uptake and chemosensitivity in multidrug-resistant MCF-7-AdrR cells. *A* and *B,* influence of PPMP on ganglioside levels and MDR1 expression. *A,* MCF-7-AdrR cells were incubated with D,L-*threo*-PPMP (5.0 μmol/L, 96 hours). Gangliosides were extracted and quantified by sialic acid derivitization. PPMP treatment did not alter cell doubling time. *B,* MDR1 mRNA levels in MCF-7-AdrR cells incubated in the absence and presence of either D,L-*threo*-PPMP or D,L-*erythro*-PPMP (15 μmol/L, 48 hours). D,L-*erythro*-PPMP did not influence cell growth; the *threo* form retarded cell growth 20%, compared with control. RNA was extracted and quantified by real-time RT-PCR. *C,* influence of GCS siRNA (100 nmol/L, 48 hours) on GCS and MDR1 mRNA expression in MCF-7-AdrR cells. LipofectAMINE alone was used as control. *D,* vinblastine uptake was determined in MCF-7-AdrR cells preincubated with D,L-*threo*-PPMP (5.0 μmol/L, 96 hours). PPMP did not alter cell growth. *E,* vinblastine cytotoxicity in MCF-7-AdrR cells preincubated with D,L-*threo*-PPMP (5.0 μmol/L, 96 hours) and exposed to vinblastine at the indicated concentrations for 3 days. Cell viability was determined by MTS assay. *Columns,* mean of triplicates from two independent experiments; *bars,* ±SD. *. $P < 0.05$; **, $P < 0.01$.

Discussion

The present study shows that GCS antisense transfection of multidrug-resistant human breast cancer cells modifies cellular lipid composition, reduces MDR1 expression, and enhances the cytotoxic effect of chemotherapeutic drugs. GCS antisense transfection decreased the levels of sphingomyelin in MCF-7-AdrR cells. Sphingomyelin is a major constituent of the external leaflet of the plasma membrane (34). Sphingomyelin, phosphatidylcholine, and proteins are laterally organized in biological membranes (35–37). These organized domains have been suggested to participate in cellular processes, such as signal transduction, membrane trafficking, and protein sorting (38). Expression of the principal component of caveolae, caveolin-1, in MCF-7-AdrR and MCF-7-AdrR/GCS antisense cells, determined by Western blot, was not affected by antisense down-regulation of GCS (data not shown), although it should be mentioned that this assay is not a good estimate of the status of cellular lipids in rafts/caveolae. However, we found that transfected cells had lower levels of gangliosides, the sialic acid–containing glycosphingolipids. Gangliosides have been shown to influence lipid order and hydration of the lipid bilayer; such changes could play an important role in modulation of transmembrane molecular events (39). Moreover, gangliosides have been shown to influence membrane fluidity (40–42). Cellular ganglioside levels decreased 4-fold in MCF-7-AdrR/GCS antisense cells compared with MCF-7-AdrR cells. Such a change could modify membrane permeability and facilitate entrance of natural-product chemotherapeutic agents such as vinblastine and paclitaxel.

In addition to their role as a structural component of the plasma membrane, gangliosides might regulate signaling events. In melanoma cells, transient ganglioside depletion by GCS inhibition reduced tumorigenic capacity (43). Gangliosides can also induce production of nitric oxide, tumor necrosis factor-α, and cyclooxygenase 2 and activate extracellular signal–regulated kinase and c-*jun*/stress-activated protein kinase kinase, p38, and nuclear factor κB (NF-κB; ref. 44). Our work showed that inhibiting the activity of GCS severely limited the expression of MDR1 and its product, P-glycoprotein. Studies have shown that the MDR1 promoter can be activated directly by anticancer agents such as vincristine, daunorubicin, doxorubicin, and colchicine (45);

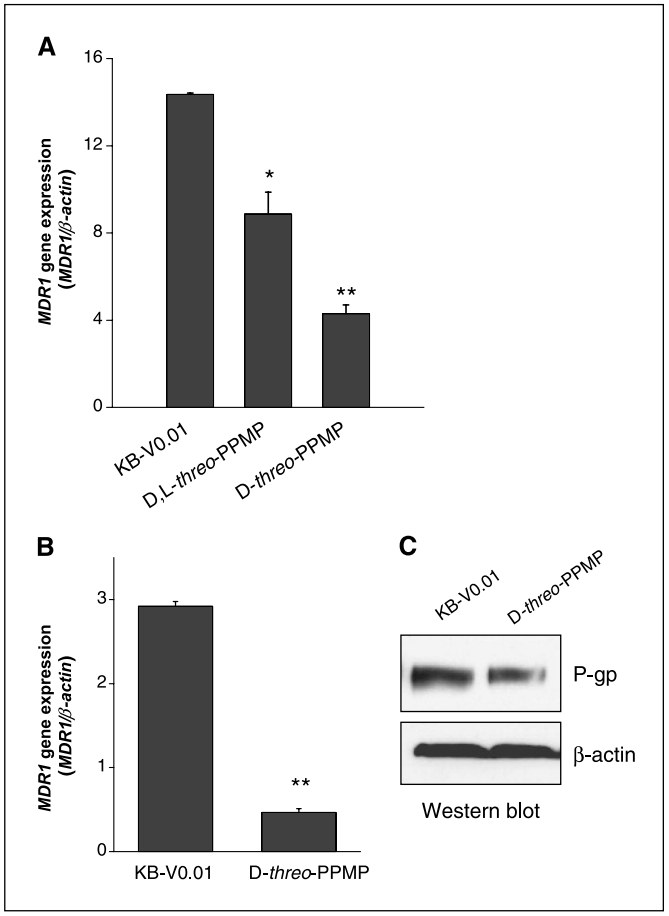

Figure 5. Influence of PPMP on MDR1 mRNA and protein (P-glycoprotein) levels in KB-V0.01 cells. *A,* MDR1 mRNA levels in KB-V0.01 cells incubated in the absence and presence of either D-*threo* PPMP (10 μmol/L, 72 hours) or D,L-*threo* PPMP (15 μmol/L, 72 hours). Measurements were made by real-time RT-PCR. Compared with control, D-*threo* PPMP and D,L-*threo* PPMP reduced cell growth by 20% and 40%, respectively. *B* and *C,* KB-V0.01 cells were grown with D-*threo* PPMP (10 μmol/L) for 7 days, and RNA and protein were extracted and used for real-time RT-PCR (*B*) and Western blot analysis (*C*). Seven-day exposure to D-*threo* PPMP, which necessitated a medium change, increased cell doubling time by twice, compared with controls. *Points,* mean from two independent experiments; *bars,* ±SD. *, $P < 0.05$; **, $P < 0.01$.

however, an association between glycolipids and the MDR1 promoter has not been clearly established. Some studies have suggested that glycolipids, in particular gangliosides, modulate multidrug resistance. For example, the up-regulation of GM3 biosynthesis in fenretinide-adapted A2780 ovarian cancer cells has been correlated with fenretinide resistance (46). In human leukemia cells, ganglioside depletion is believed to account for PDMP-mediated reversal of multidrug resistance, and GM3 and GD3 are thought directly involved via modulation of P-glycoprotein function through phosphorylation (47). Shabbits et al. (48) showed a relationship between drug transport and ceramide metabolism. Other support for a link between glycolipids and multidrug resistance may be found in the PPMP-modulated expression of MDR1 mRNA in SKOV3/AdrR human ovarian cancer cells (49), in KBV200 cells (50), and in the decreased efflux of [^{14}C]paclitaxel and [^{3}H]vincristine in a neuroblastoma cell model (51). Results of other studies show that verapamil, an antihypertensive formerly used clinically as a P-glycoprotein antagonist (52), limits the expression of MDR1 in human leukemia cells (53). Our group showed that verapamil, tamoxifen, and cyclosporine A block glucosylceramide formation and resultant downstream cerebroside and ganglioside biosynthesis in drug-resistant cancer cells (54).

Previously, we showed that doxorubicin treatment of MCF-7-AdrR/GCS antisense cells enhanced the production of ceramide (16). In the present study using radiolabeling, ceramide buildup was not evident in MCF-7-AdrR/GCS antisense cells challenged with either vinblastine or paclitaxel (Fig. 1*A*); however, lipid mass analysis by TLC char clearly showed elevated ceramide levels in drug-challenged GCS antisense transfectants (Fig. 1*B*). Failure of radiolabeling techniques to accurately portray mass is not uncommon. More importantly however is the apparent dual role that GCS antisense transfection and/or GCS blockade play in sensitizing multidrug-resistant cancer cells to chemotherapy. From our experiments, it is evident that GCS antisense (i) down-regulates expression of MDR1 and (ii) promotes ceramide buildup in cells that would otherwise scavenge ceramide via elevated GCS activity. This one-two punch could be of benefit in cancer treatment.

The present results suggest that glycolipids participate in MDR1 expression directly or via activation of a specific transcription factor. In a recent study, Bentires-Alj et al. (55) showed that NF-κB inhibition increased cellular uptake of daunorubicin and reduced expression of MDR1 mRNA and protein (P-glycoprotein) in colon cancer cells. NF-κB complexes can bind at a consensus NF-κB binding site in the first intron of the human *MDR1* gene. Moreover, NF-κB can transactivate an MDR1 promoter luciferase construct (55).

In conclusion, our work shows that limiting GCS activity by either GCS antisense transfection, siRNA transfection, or PPMP treatment down-regulates the expression of P-glycoprotein. It should be noted, however, that drug resistance through enforced overexpression of GCS in wild-type MCF-7 cells, using a retroviral tetracycline-on expression system, did not rely on P-glycoprotein induction (13) but rather on ceramide scavenging. In addition, GCS antisense transfection retards clearance of ceramide generated in response to chemotherapeutics such as paclitaxel. Therefore, ceramide-signaled death cascades and depletion of cellular P-glycoprotein are likely contributory to heightened chemosensitivity in MCF-7-AdrR/GCS antisense. We propose that overexpression of GCS contributes to chemotherapy resistance by

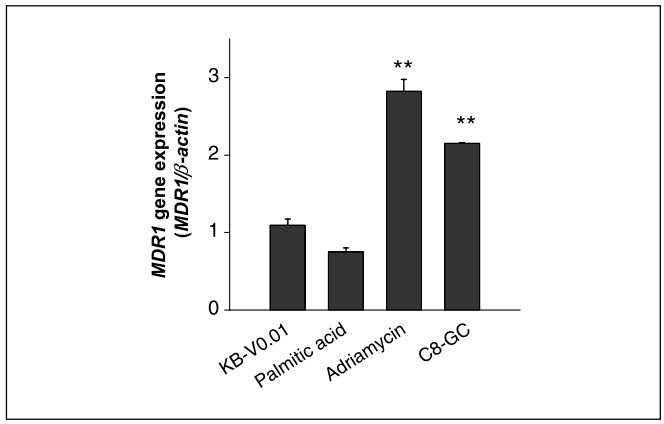

Figure 6. Influence of C8-glucosylceramide supplement on MDR1 expression in KB-V0.01 cells. Cells were incubated for 48 hours with either C8-GC (30 μg/mL), Adriamycin (0.5 μmol/L) used as positive control, or palmitic acid (10 μg/mL) used as lipid control. RNA was extracted and analyzed by real-time RT-PCR. C8-GC was not cytotoxic at the employed dose. *Columns,* mean from two independent experiments; *bars,* ±SD. **, $P < 0.01$.

enhancing levels of cerebrosides and/or gangliosides that could promote the expression of MDR1. Doxorubicin-activated expression of GCS in MCF-7 drug-sensitive cells (56) lends support to this novel slant on the metabolism of ceramide.

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SUPPORTING DOCUMENTS

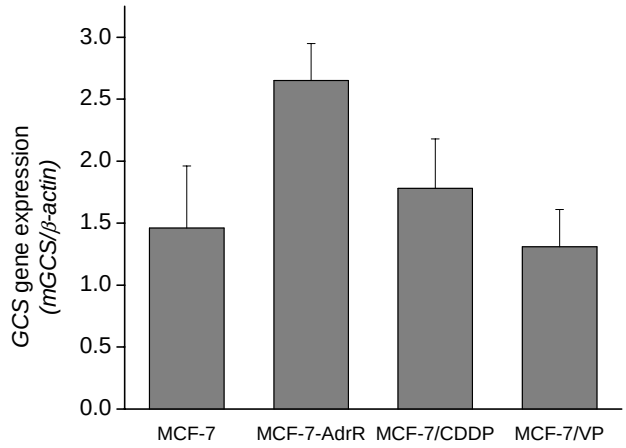


Figure 1: GCS gene expression in resistant breast cancer cells. RNA was extracted and analyzed by real-time RT-PCR.

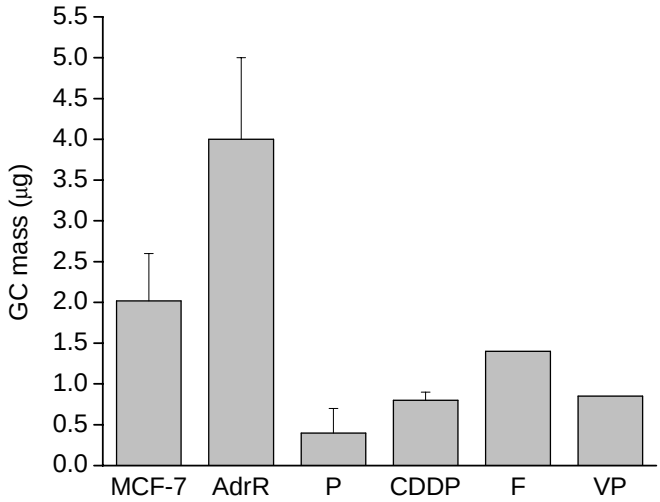


Figure 2: Determination of GC mass in resistant breast cancer cells. Total lipids extracted from washed cells, were analyzed by TLC (250 μg lipid/lane). The solvent system contained chloroform/methanol/ammonium hydroxide (70:20:4, v/v/v), visualization was by sulfuric acid char, and GC quantization was by scanning densitometry using a GC standard curve (0.5-5.0 μg).

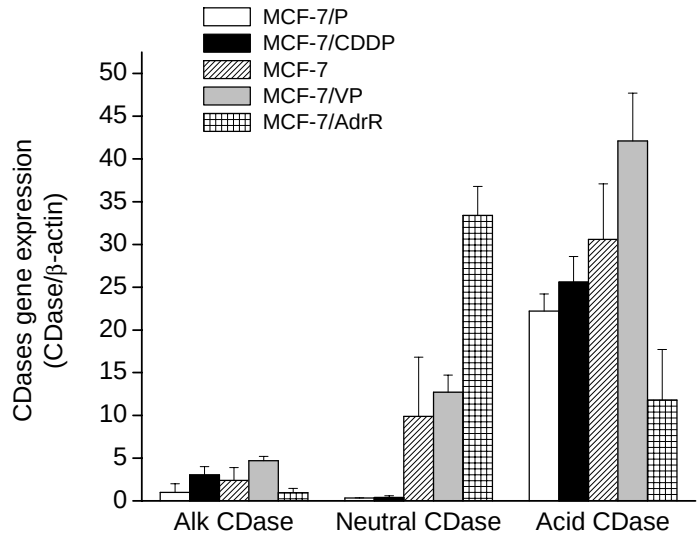


Figure 3: Expression of ceramidase isoforms in MCF-7 and drug resistant MCF-7 cells. Total RNA from MCF-7/P, MCF-7/CDDP, MCF-7, MCF-7/VP, and MCF-7/AdrR cells was extracted and analyzed by real-time RT-PCR.

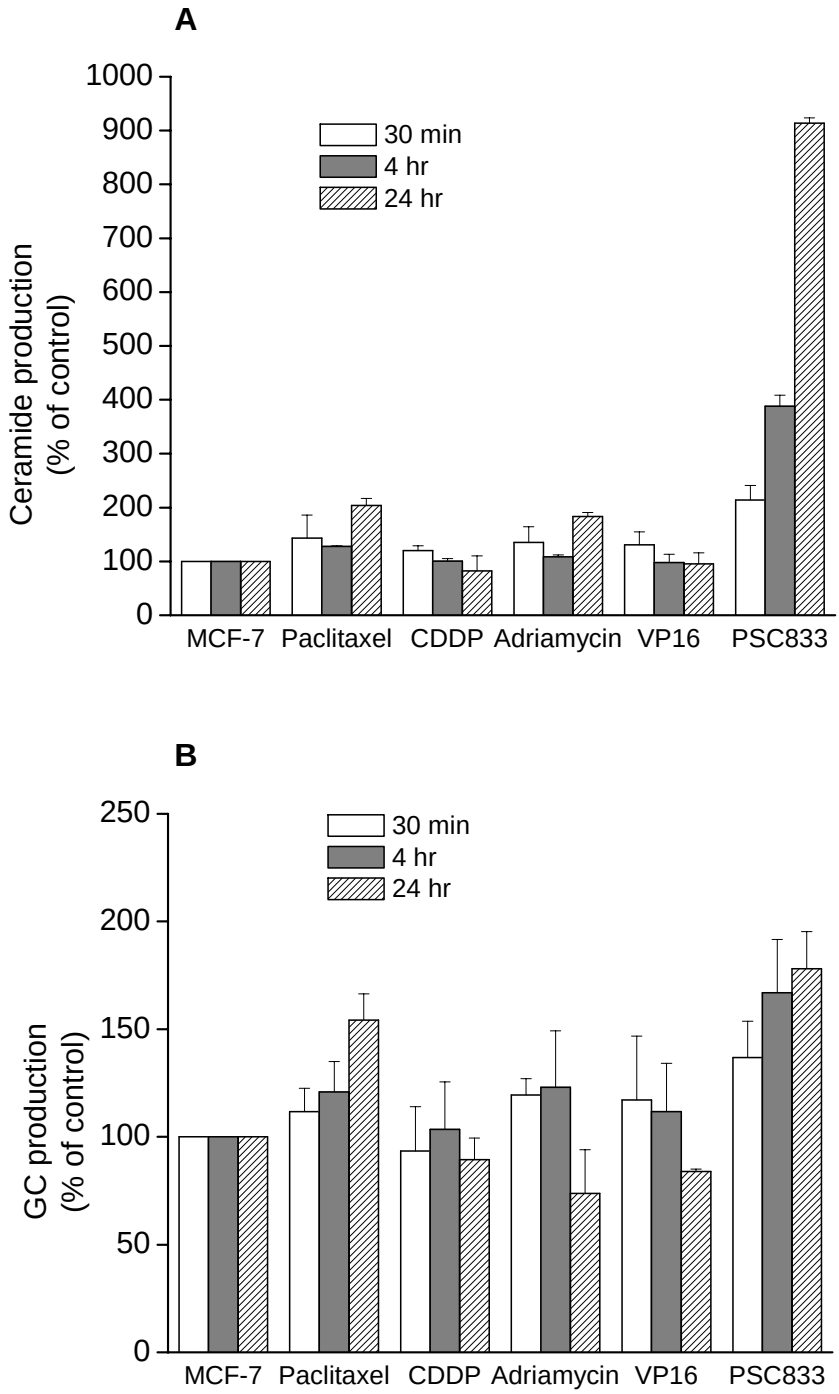


Figure 4: Influence of anticancer drugs on ceramide and glucosylceramide production.
MCF-7 cells were seeded in 6 well-plates (750,000 cells/well) with or without paclitaxel (1.5 μM), CDDP (10 μM), Adriamycin (2.5 μM), VP16 (1.0 μM), and SDZ PSC 833 (5.0 μM) in culture medium containing 2.0 μCi/ml [^{3}H]palmitic acid for the indicated times. Ceramide (A) and GC (B) were resolved from total lipid extracts by TLC.

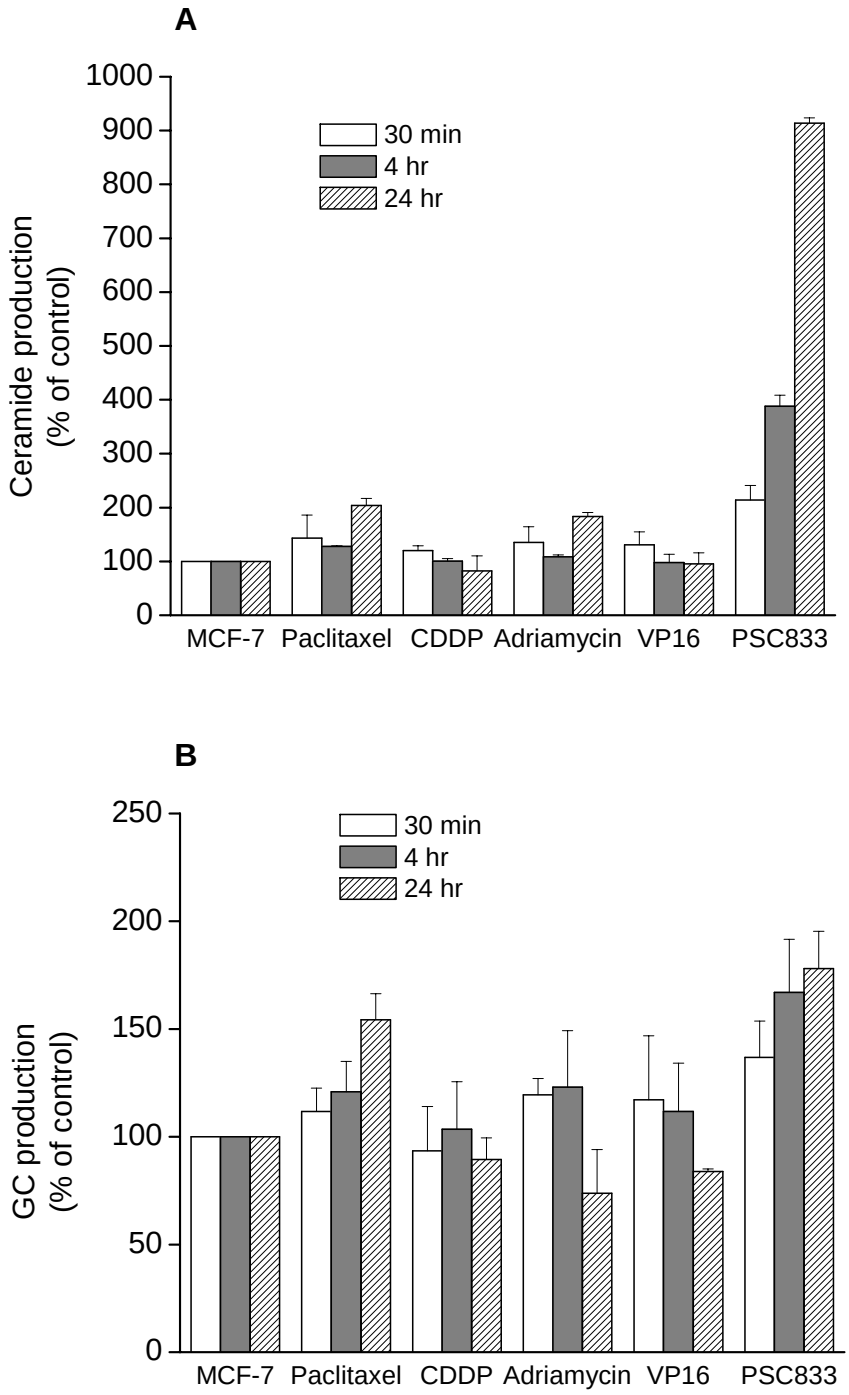


Figure 5: Influence of anticancer drugs on ceramide and glucosylceramide production.
MCF-7 cells were seeded in 6 well-plates (750,000 cells/well) with or without paclitaxel (1.5 μM), CDDP (10 μM), Adriamycin (2.5 μM), VP16 (1.0 μM), and SDZ PSC 833 (5.0 μM) in culture medium containing 2.0 μCi/ml [^{3}H]palmitic acid for the indicated times. Ceramide (A) and GC (B) were resolved from total lipid extracts by TLC.

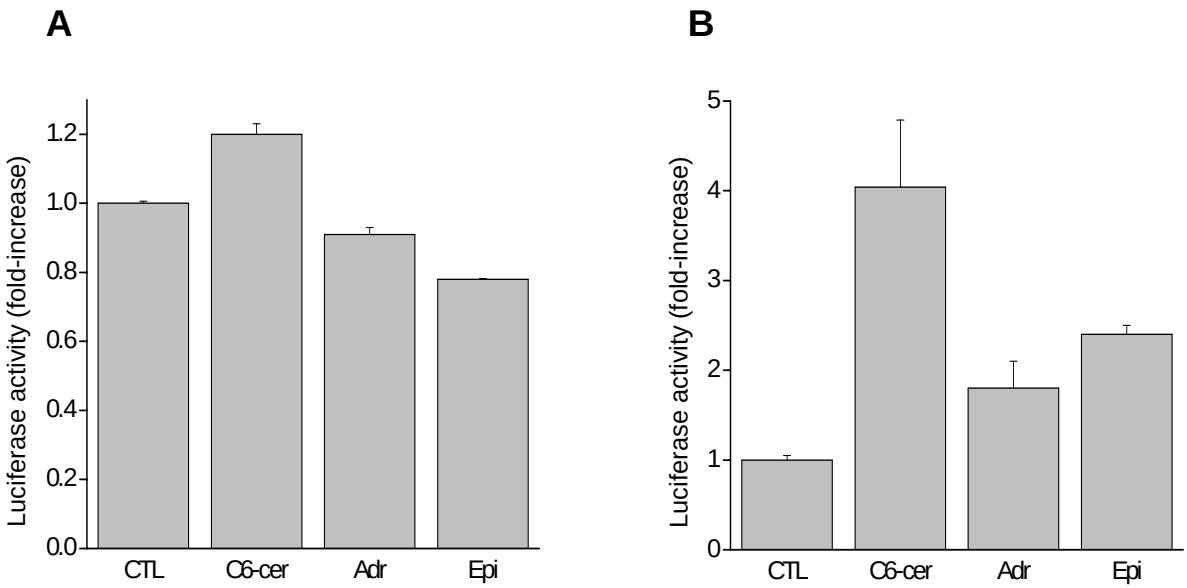


Figure 6: GCS promoter activity analysis. After 24 hr transfection with full-length GCS promoter plasmid (pGCS-Luc1, 4 μg DNA/well), MCF-7 cells were maintained in 5% FBS RPMI-1640 medium containing adriamycin (0.5 μM), C6-ceramide (5.0 μM), or epirubicin (0.5 μM) for **A**, 4hr and **B**, for 48 hr. Luciferase activity was then determined. The control represents MCF-7 cells transfected with pGCS-Luc1 without treatment. C6-cer: C6-ceramide, Adr: adriamycin, Epi: epirubicin.

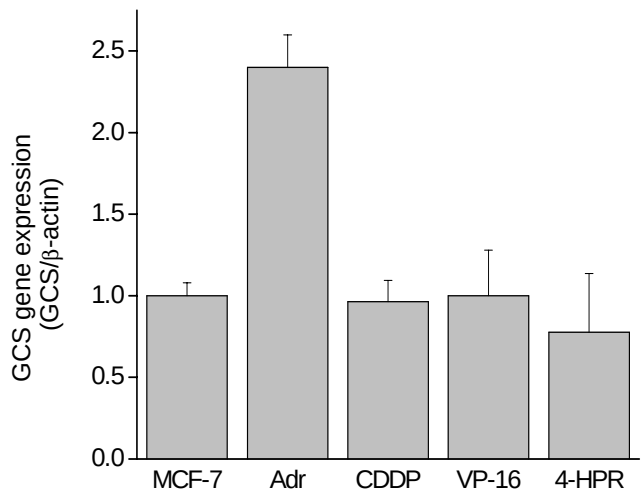


Figure 7: The influence of anticancer drugs on GCS gene expression. MCF-7 cells were seeded in 6-cm dishes and the following day treated with adriamycin (2.5 μM), etoposide (0.5 μM), CDDP (10 μM), or 4-HPR (5.0 μM). Control dishes received ethanol (0.1% final concentration). After 48 hr, RNA was extracted and analyzed by real-time RT-PCR.

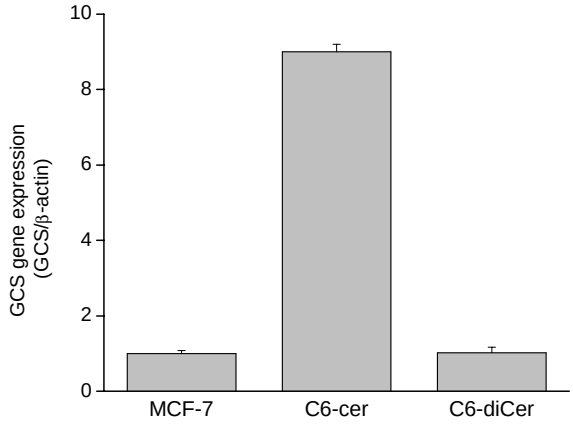


Figure 8: The influence of ceramide on GCS gene expression. MCF-7 cells were treated with C6-ceramide (5.0 μM) or C6-dihydroceramide (5.0 μM) for 48 hr. RNA was extracted and analyzed by real-time RT-PCR.

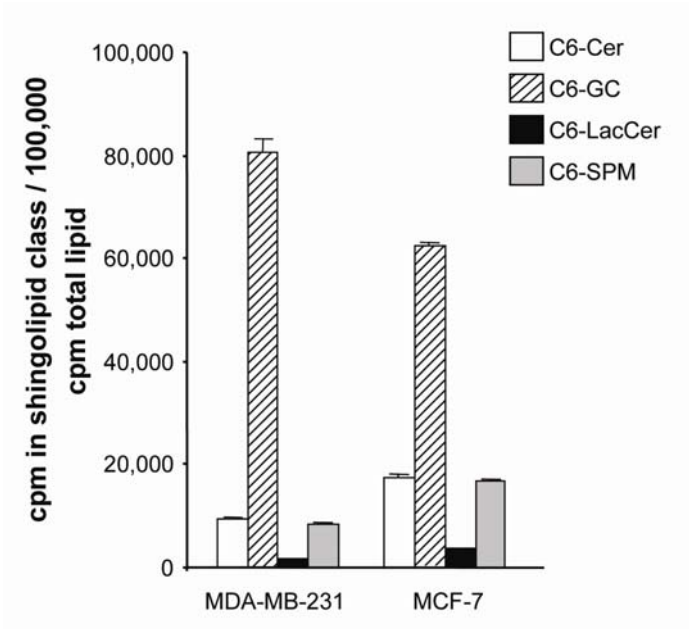


Figure 9: Metabolism of [^{14}C]C6-ceramide in MCF-7 and MDA-MB-231 cells. Cells in 6-well plates were grown with [^{14}C]C6-ceramide (500,000 cpm/ml, 5 μg/ml) for 24 h, after which total lipids were extracted and analyzed by TLC for radiolabeled free C6-ceramide, C6-GC, C6-SPM and C6-LacCer.

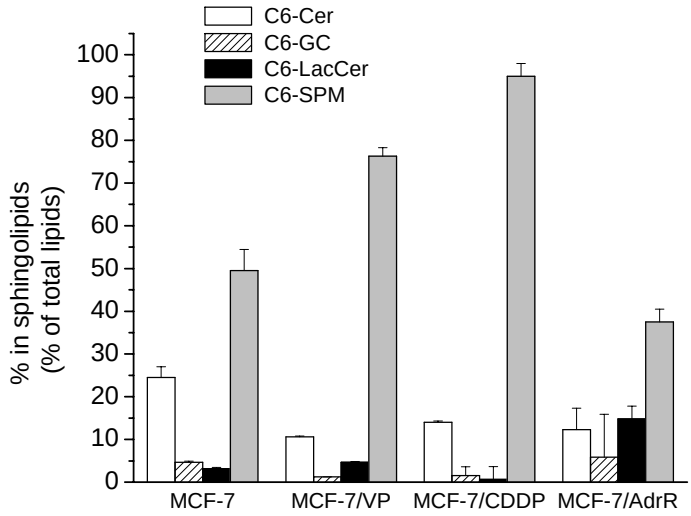


Figure 10: Metabolism of [^{14}C]C6-ceramide in MCF-7 and drug resistant MCF-7 cells. Cells in 6-well plates were grown with [^{14}C]C6-ceramide (84,500 cpm/well, 0.2 μg/ml) for 24 h, after which total lipids were extracted and analyzed by TLC for radiolabeled free C6-Cer, C6-GC, C6-LacCer, and C6-SPM.

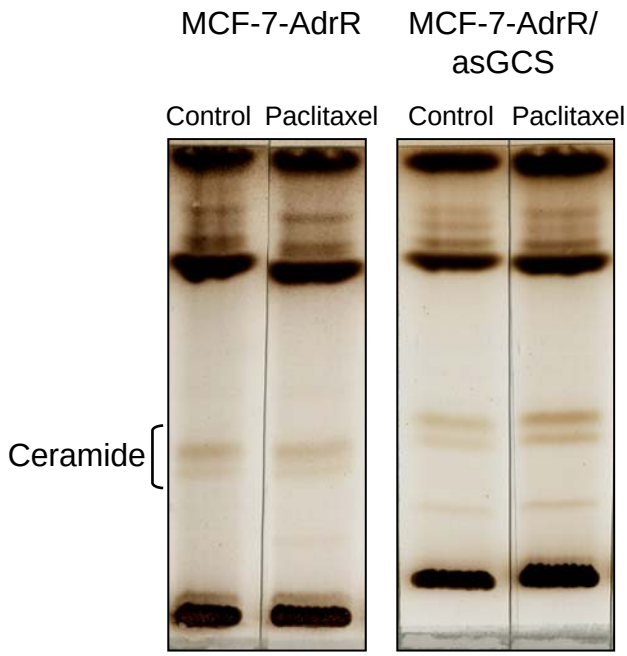


Figure 11: Influence of paclitaxel on ceramide levels in MCF-7-AdrR and MCF-7-AdrR/asGCS cells. Total cellular lipids were extracted, and ceramide was resolved by TLC (chloroform/acetic acid; 90:10, v/v). The chromatogram was sprayed with sulfuric acid. 880 μg total lipid was loaded in each lane.

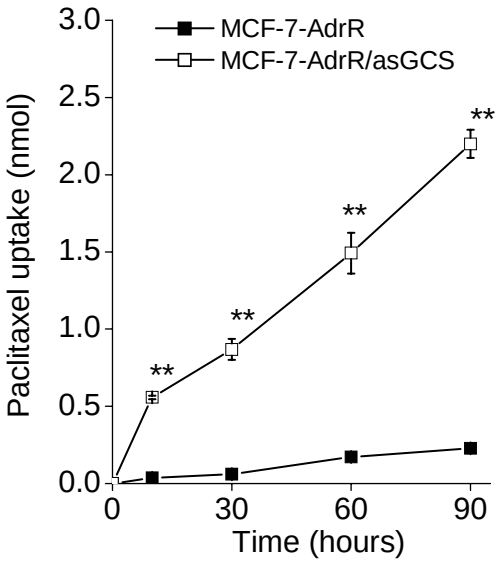


Figure 12: Uptake of paclitaxel in MCF-7-AdrR and MCF-AdrR/asGCS cells. Data represent mean ± S.D. from two independent experiments (** p<0.01).

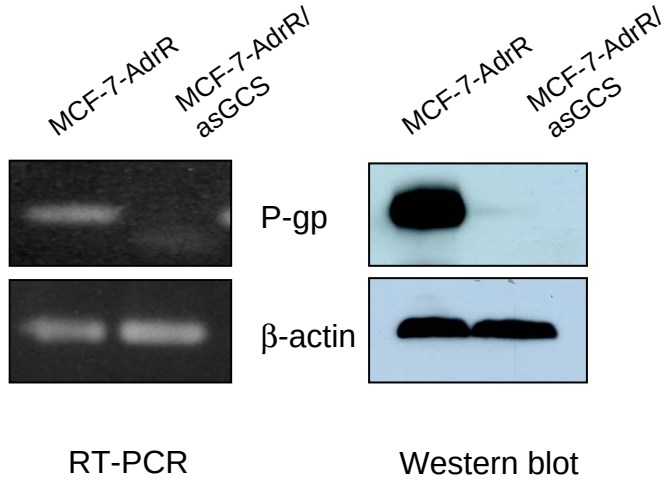
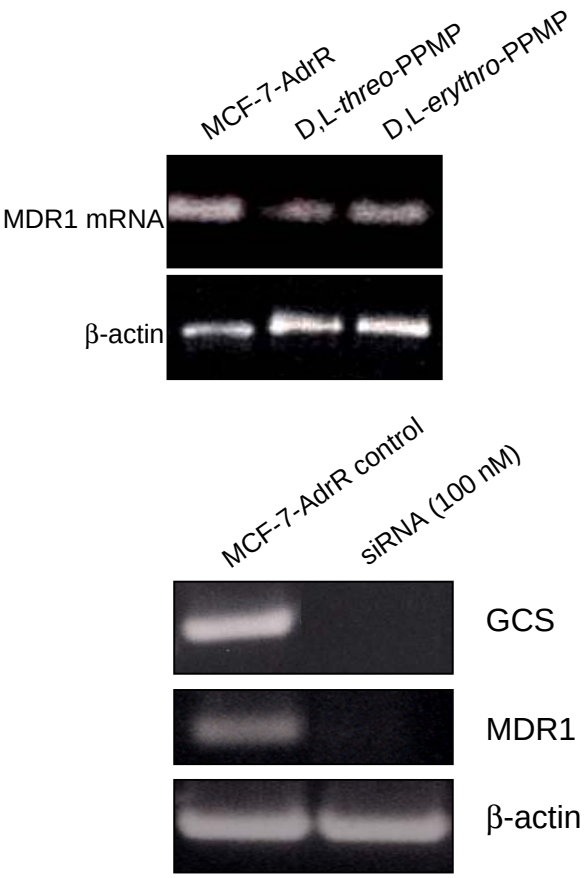


Figure 13: P-gp expression in MCF-7-AdrR and MCF-7-AdrR/asGCS cells. MCF-7-AdrR and MCF-7-AdrR/asGCS cells were grown to 70% confluence, and RNA and protein were extracted and used for RT-PCR and Western blot analysis. The C219 murine monoclonal antibody against human P-gp was used for the Western blot.



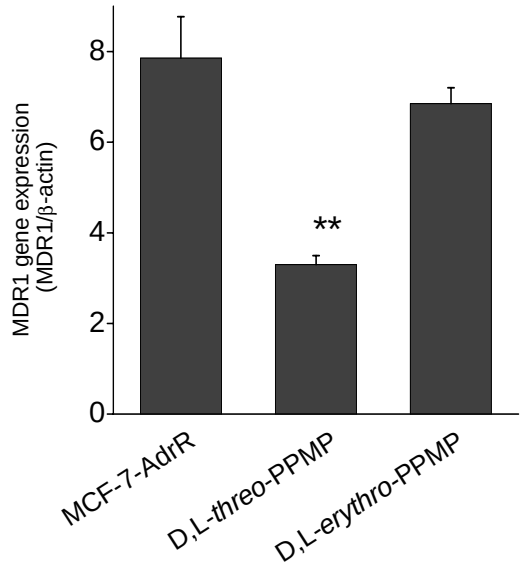
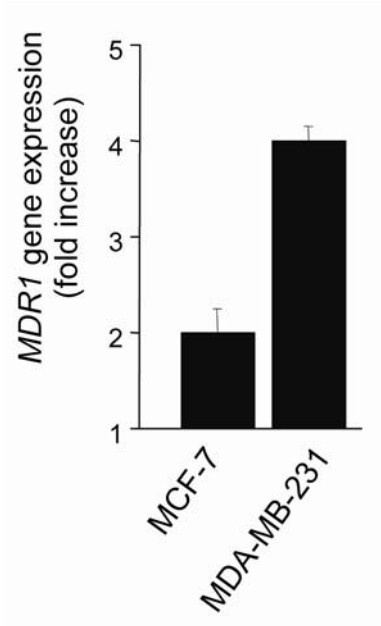


Figure 14. Influence of PPMP and GCS siRNA on MDR1 gene expression in multidrug resistant MCF-7-AdrR cells. ***A*, *B.*** MDR1 mRNA levels in MCF-7-AdrR cells incubated in the absence and presence of either D,L-*threo*-PPMP or D,L-*erythro*-PPMP (15 μM, 48 h). RNA was extracted, analyzed by RT-PCR (A), and quantified by real-time RT-PCR (B). ***C.*** Influence of GCS siRNA (100 nM, 48 h) on GCS and MDR1 mRNA expression in MCF-7-AdrR cells. Lipofectamine alone was used as control.

Figure 15: Influence of C8-GC on MDR1 expression in MCF-7 and MDA-MB-231 cells. Cells (500,000) were seeded into 6-cm dishes and the following day supplemented with C8-GC (10 μg/ml medium). After 72 h, total RNA was extracted and analyzed by qPCR. Fold increases in MDR1 were calculated using actual number of gene copies per unit β-actin expression.



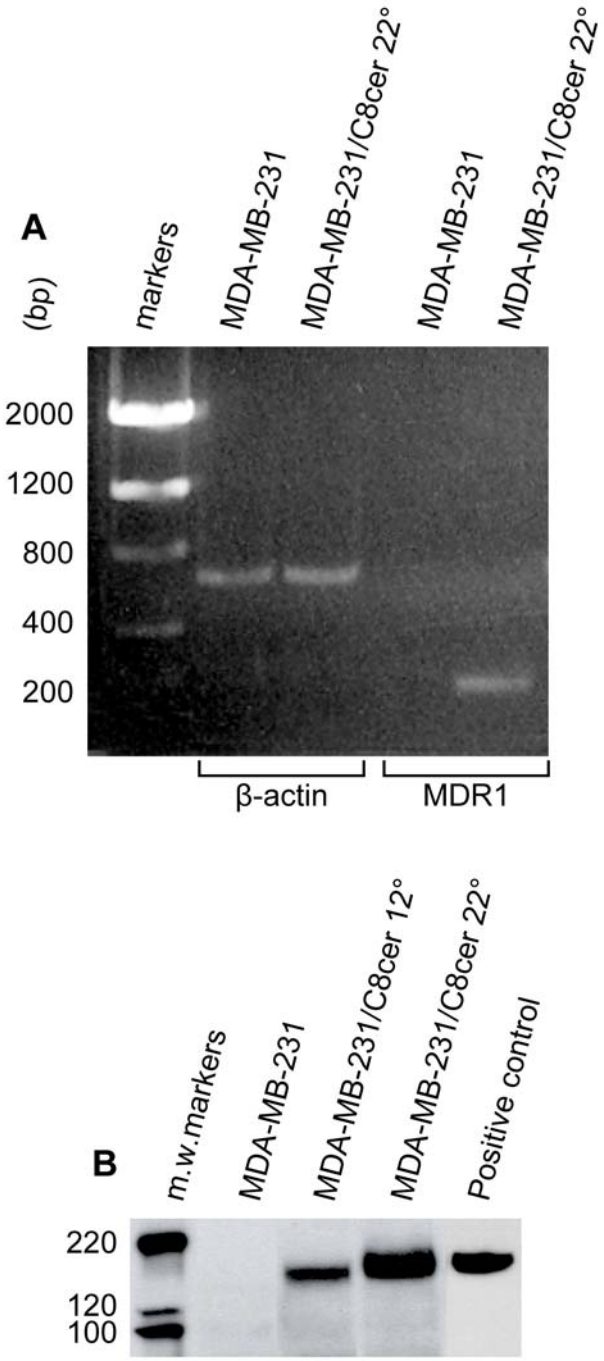


Figure 16: The influence of chronic exposure to C8-ceramide on MDR1 mRNA, P-gp levels in MDA-MB-231 cells. **(A)** MDR1 mRNA levels by RT-PCR in MDA-MB-231 cells and in high passage C8-ceramide cells (MDA-MB-231/C8cer, passage 22). Samples were subjected to RT-PCR analysis (0.5 µg RNA/tube) and products were resolved on 1% agarose gels. β-actin was employed as housekeeping gene. **(B)** P-gp levels by Western blot in MDA-MB-231 and in MDA-MB-231/C8cer cells at passages 12 and 22. Aliquots (100 µg cell protein) were electrophoresed for Western blot analysis of P-gp (C219 antibody). KB-ChR-8-5 (colchicine-resistant human epidermoid carcinoma) cell protein (50 µg) was used as a positive control for P-gp.

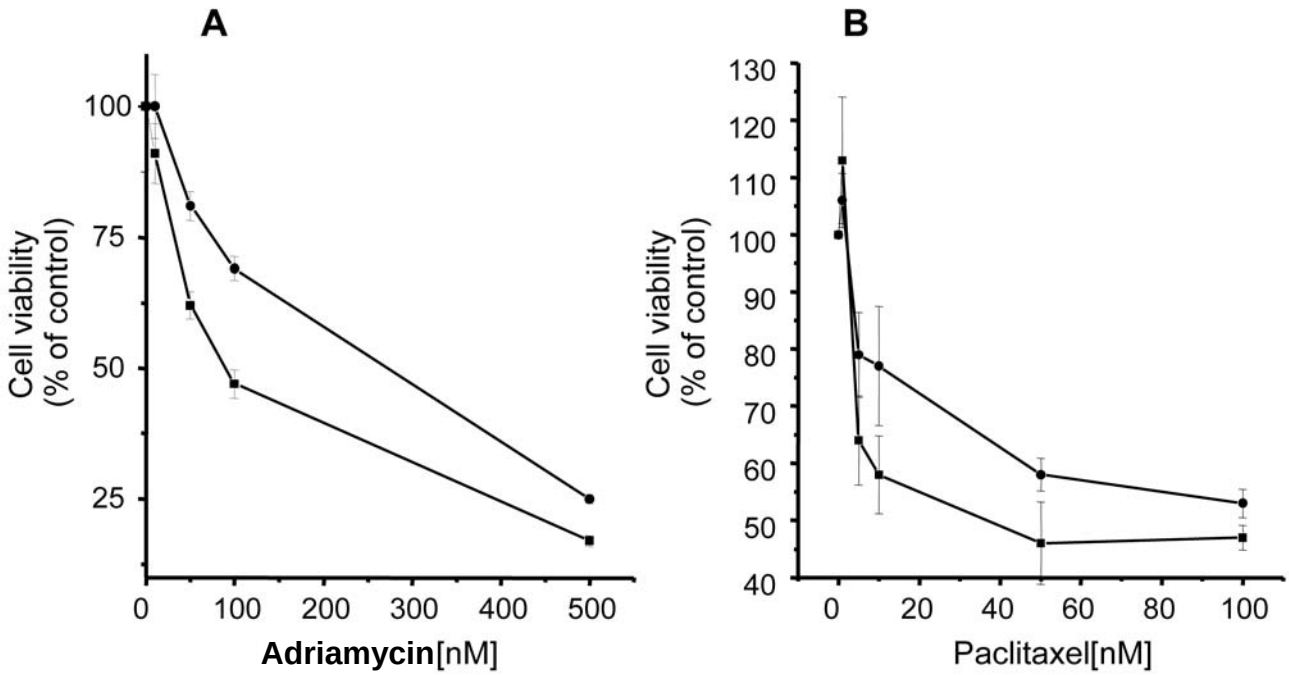


Figure 17: Doxorubicin and paclitaxel sensitivity in MDA-MB-231 (■) and MDA-MB-231/C8cer cells (●). MDA-MB-231/C8cer cells (passage 17) were used in the chemosensitivity assays, (A) adriamycin and (B) paclitaxel. C8-ceramide was not in the medium during the experiment. Cell viability was determined by MTS assay.